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Clinical and Experimental

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The Journal of Laboratory and Clinical Medicine

VOL. 43 APRIL, 1954 No. 4

CLINICAL AND EXPERIMENTAL

THE EFFECTS OF MASSIVE GASTROINTESTINAL HEMORRHAGE ON HEMOSTASIS

I. THE BLOOD PLATELETS

JANE F. DESFORGES, M.D., FREDERICK S. BIGELOW, M.D., AND THOMAS C. CHALMERS, M.D.

BOSTON, MASS.

THE great majority of patients with massive upper gastrointestinal hemorrhage respond promptly to blood replacement and early feeding. Some patients, however, continue to bleed or have recurrent massive hemorrhage after adequate therapy has been undertaken. In the fatal cases, each recurrent bout of hemorrhage is apt to be more severe than the preceding one. Furthermore, it has been observed that when surgery is undertaken after more than two days of bleeding, the tissues bleed profusely. These clinical observations led to the studies herein reported of hemostatic mechanisms in patients with massive gastrointestinal hemorrhage. Observations of platelets, prothrombin complex, and the thrombin-fibrinogen reaction as well as tests to evaluate vascular integrity were carried out during periods of hemorrhage and convalescence. In this paper are presented the observed changes in blood platelets and platelet function and the correlation of these changes with the clinical state of the patient.

METHODS AND MATERIALS

The platelet counts were determined by the method of Pohle.[1] In this laboratory, 96 per cent of the normal values fall between 174,000 and 524,000 per cubic millimeter,

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with the median at 260,000 per cubic millimeter and the mean at 283,000 per cubic millimeter (Fig. 1). Because of the wide range and skew distribution of the platelet counts, median values instead of the means are used throughout most of this paper. Platelet stickiness was calculated by a modification of the method of Wright[2] with constant pH maintained by equilibration with a gas mixture of 5 per cent CO_2–95 per cent O_2. Fluid volume of the clot was measured by the method of Aggeler and co-workers.[3] Bleeding time was observed by lancing the ear lobe with a sharp scalpel blade to a depth of 2 mm. and a width of 2 mm. and then carefully blotting the free flowing blood until bleeding ceased. The upper limit of normal by this technique is six minutes. Capillary fragility was determined by applying a sphygmomanometer to the arm at a pressure of 100 mm. of mercury for ten minutes. Five minutes after the cuff was removed, the forearm and hand were examined for petechiae and the test interpreted as negative to 4 plus according to the number and distribution of petechiae. Electrophoresis of platelets was studied according to the method of Bigelow.[4]

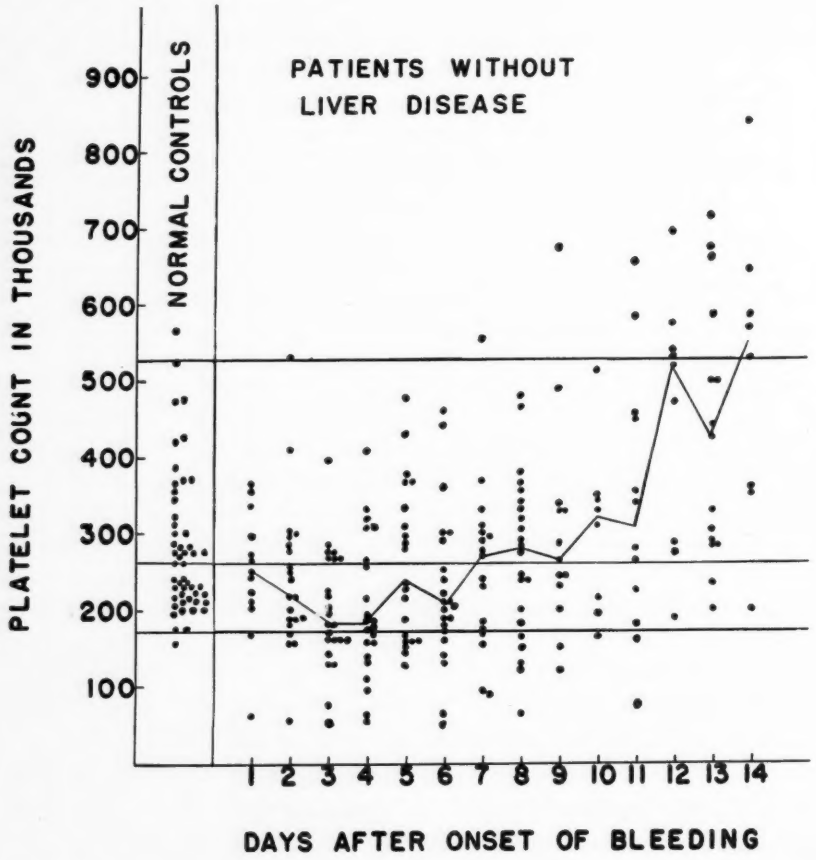

Fig. 1.—The platelet counts of the patients without liver disease are charted according to the day after the onset of bleeding. The distribution of platelet counts of 50 normal controls is charted at the left. The horizontal lines represent the limits and median of the normal data. The irregular line is the median of the patients' counts.

The patients studied were those admitted to the Boston City Hospital Medical Services with massive upper gastrointestinal hemorrhage. Whenever possible, determinations were performed at the time of entry, prior to the start of therapy. Thereafter, the studies were usually performed in the morning. Since blood coagulation has been shown

to be abnormal in the presence of liver disease,[5, 6] the patient material was divided into two groups depending on the presence or absence of chronic liver disease. Forty-five patients were included in the group with no clinical or pathologic evidence of cirrhosis. Abnormal bromsulfalein retention was occasionally noted in these patients during active hemorrhage, but it always returned to normal early in convalescence. Twenty-seven patients with cirrhosis were observed during 45 episodes of massive upper gastrointestinal hemorrhage. Only those patients who showed evidence of extensive liver disease either by clinical findings or by pathologic examination were included. Studies of eighteen patients in whom the evidence for liver disease was equivocal or incomplete were excluded from the results. Platelet counts were also obtained as controls in 24 patients hospitalized for decompensated cirrhosis without hemorrhage and in 15 patients with previously decompensated cirrhosis who were receiving care in the outpatient department and were relatively free of symptoms.

The incidence of abnormalities most commonly associated with severity of hemorrhage is presented in Table I. Except for a lower than usual mortality rate in those without liver disease, the material was typical of that commonly encountered in a charity hospital.

TABLE I. PATIENT MATERIAL CLASSIFIED ACCORDING TO THE USUAL CRITERIA OF SEVERITY OF HEMORRHAGE

	NO KNOWN LIVER DISEASE	CIRRHOSIS
Total patients	45	27
Total episodes of hemorrhage	46	45
Number with shock	13	24
Lowest hematocrit		
Above 30	12	13
21-30	20	25
Below 21	14	7
Number not transfused	11	5
1-3 transfusions	19	13
4-6 transfusions	9	13
More than 6 transfusions	7	14
Preoperative deaths	1	8
Emergency surgery	8	4
Postoperative deaths	1	3
Total deaths	2	11

RESULTS

Patients Without Liver Disease.—In Fig. 1, all the platelet counts performed on patients without known liver disease have been plotted against time after onset of the hemorrhage. At the left for comparison are the counts done in 50 normal controls. The plotted medians for the patients' counts dropped below the normal median during the first week and then continued to rise above normal up to the fourteenth day after onset. The number of patients followed after that time was too few to be charted. Counts below the lower limit of normal occurred up to the eleventh day after the onset of bleeding. To demonstrate more clearly the relationship between the platelet response and the course of the patient, the counts were replotted in Fig. 2, using the apparent last day of hemorrhage rather than the day of onset as the reference point. There was a distinct grouping of the lowest counts in the period during hemorrhage and for three days following cessation. Ninety-six per cent of the abnormally low counts

occurred before the sixth day. It is evident that those platelet counts found to be low in some of the patients many days after the onset of hemorrhage were associated with continued bleeding.

The abnormal drop in platelet counts during and soon after cessation of bleeding, and the rise to levels above normal during convalescence appear to be significant. To eliminate any statistical bias attributable to more frequent determinations in patients with abnormal counts, the data in Table II were compiled. Each patient's counts during each of four successive four-day periods were averaged. The mean of the patient's means for each period was thus uninfluenced by the frequency of the determinations. These figures show a gradual rise in platelet count following cessation of bleeding. The differences between the periods are highly significant.

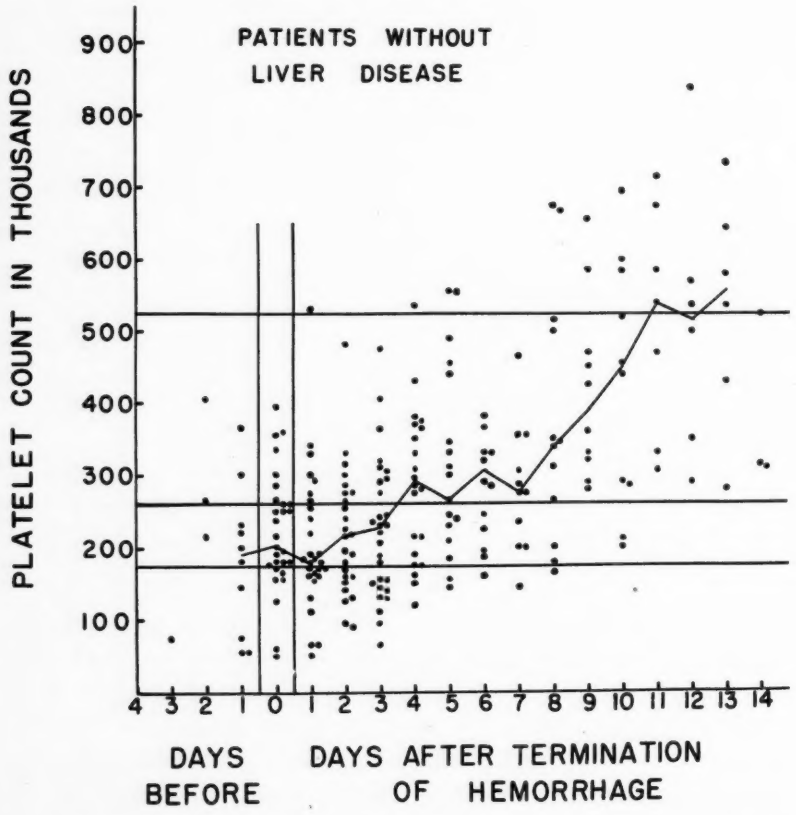

Fig. 2.—The platelet counts of the patients without liver disease are charted according to the time of cessation of bleeding. The horizontal lines represent the limits and median of the normal data. The irregular line is the median of the patients' counts.

That the patients with excessively low platelet counts were capable of attaining normal ones is shown in Fig. 3. Six of the 45 patients had one or more counts below 100,000. In all six, later determinations were within or above the normal range.

TABLE II. PATIENTS WITHOUT KNOWN LIVER DISEASE: MEAN OF THE MEAN DAILY COUNTS IN EACH OF 4 PERIODS DURING AND AFTER CESSATION OF HEMORRHAGE

TIME IN DAYS IN RELATION TO LAST DAY OF HEMORRHAGE	NUMBER OF PATIENTS	MEAN × 1,000	STANDARD DEVIATION × 1,000	P*
−2 to +2	42	213	78	
3 to 6	33	263	74	.01
7 to 10	21	359	106	.02
11 to 14	15	456	120	.02

*Probability of the observed difference between the two means occurring by chance.

The correlation coefficient between the patients' lowest hematocrit and lowest platelet count was +0.10. There was a similar complete lack of correlation between the lowest and highest counts and the presence or absence of shock.

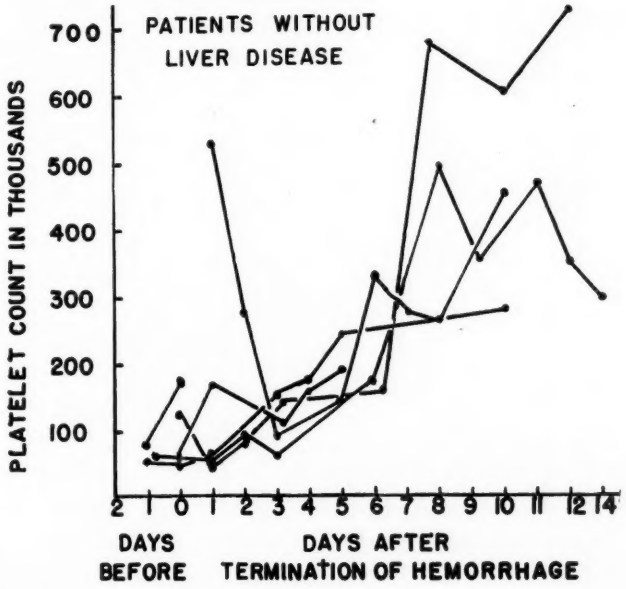

Fig. 3.—Serial platelet counts of patients who had one or more values below 100,000 per cubic millimeter are charted according to the time of cessation of hemorrhage.

In an effort to identify a possible platelet depressing factor which might be free in the circulation early in the course of hemorrhage, two patients were venesected of 500 ml. of blood within a day after entry, using ACD anticoagulant. The plasma was separated and stored at 4° C. until two weeks after cessation of hemorrhage. It was then transfused back into the patients and the platelet counts carefully observed first at hourly and then at daily intervals. In neither case was any change seen.

Patients With Cirrhosis.—The response of the platelet count to hemorrhage in the patients with cirrhosis is shown in Fig. 4. Following the onset of hemorrhage the median value fell below that of a similar group of patients without hemorrhage and remained lower for nine days. The majority of these

patients continued to bleed while being studied. While no significant difference was noted in the determinations done on these patients while still bleeding, it was found that when the values were grouped according to the days following cessation of bleeding (not charted), the number of low counts in the later period was diminished. Platelet counts below 50,000 were observed only during the first three days after hemorrhage, and after the fifth day only two of 33 determinations were found to be below 100,000 per cu. mm. Thus, gastrointestinal hemorrhage is associated with low platelet counts in patients with and without cirrhosis. In the former group the counts are much lower and the rise much slower and less complete.

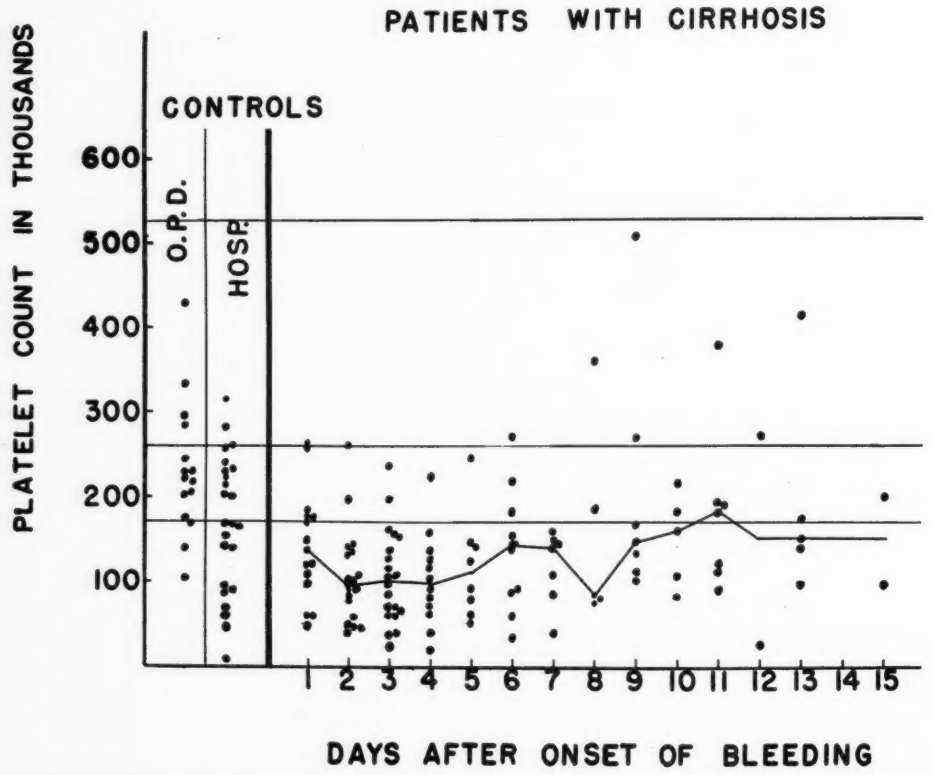

Fig. 4.—The platelet counts of the patients with cirrhosis are charted according to the day after the onset of bleeding. The two control groups consist of fairly well compensated patients being followed in the outpatient department and of sick patients in the hospital with cirrhosis but without any evidence of bleeding. The horizontal lines represent the limits and median of the platelet count in normals.

To eliminate any possible error introduced by more frequent determinations on some patients, the data in Table III were constructed in the same manner as Table II. They illustrate that the mean count also rose significantly after cessation of bleeding in the patients with cirrhosis but that there was a distinct delay when compared with the patients without liver disease. Thus there was no significant rise until the 7- to 10-day period. There was no relation between platelet count and either the severity of the patient's blood loss

anemia or the presence or absence of shock. The mean early counts in those patients who died are compared to those of the survivors for the same period in Table IV. The difference between the two is not significant.

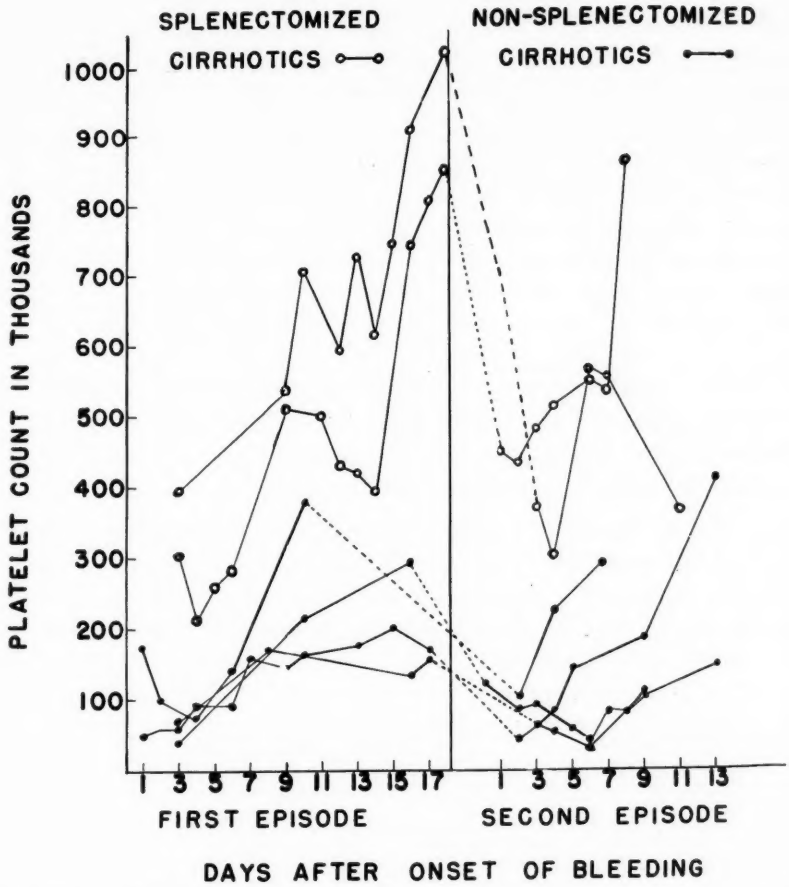

Fig. 5.—Serial platelet counts following the onset of hemorrhage are charted during repeated episodes of bleeding in patients with cirrhosis after splenectomy as compared with similar patients with spleens.

To investigate the possible role of the spleen in the response of platelets to hemorrhage, patients with cirrhosis in whom splenectomy had been performed were observed during and after episodes of gastrointestinal hemorrhage. In Fig. 5, the course of platelet counts in 2 of these patients following repeated bleeding episodes has been charted. For comparison, the figure includes four nonsplenectomized patients with severe liver disease in whom repeated episodes of gastrointestinal hemorrhage occurred. The interval between hemorrhages varied in each case. The patients with splenectomy had a similar type of platelet response, but the original and lowest values were at a much higher level. Comparison of the bone marrow smears from a patient with splenectomy and a patient with severe liver disease without splenectomy

TABLE III. PATIENTS WITH CIRRHOSIS. MEAN OF THE MEAN DAILY COUNTS IN EACH OF 4 PERIODS DURING AND AFTER CESSATION OF HEMORRHAGE

TIME IN DAYS IN RELATION TO LAST DAY OF HEMORRHAGE	NUMBER OF PATIENTS	MEAN × 1,000	STANDARD DEVIATION × 1,000	P*
−4 to +2	21	111	52	
3 to 6	10	116	37	.01
7 to 10	10	206	78	

*Probability of the observed difference between the two means occurring by chance.

TABLE IV. PATIENTS WITH CIRRHOSIS. COMPARISON OF THE MEAN COUNTS DURING BLEEDING OF PATIENTS WHO SURVIVED AND THOSE WHO DIED OR HAD EMERGENCY OPERATIONS

	NUMBER OF PATIENTS	MEAN × 1,000	STANDARD DEVIATION × 1,000	P*
Survived	10	92	39	.08
Death or operation	11	134	63	

*Probability of the observed difference between the two means occurring by chance.

early in the course of hemorrhage revealed megakaryocytes to be plentiful in both. However, while the splenectomized patient had megakaryocytes of normal appearance, the other had megakaryocytes with vacuolated and hyaline-like cytoplasm without peripheral platelet patches.

Relation of Platelet Count to Capillary Fragility, Bleeding Time, Clot Retraction, and Electrophoretic Mobility.—Six of 26 tourniquet tests were positive during 14 episodes of hemorrhage in cirrhotics. The mean platelet count coincident with the positive tests was 105,000 per cu. mm. and with the negative tests, 95,000. There also appeared to be no correlation between the results of the test and the presence or absence of shock or active bleeding.

Bleeding time was found abnormal (by one minute) in 2 of 21 tests in patients without liver disease, and in 15 of the 64 determinations in the patients with cirrhosis. In the latter group seven were longer than ten minutes. There was a lack of correlation between the long bleeding times and the low platelet counts, and similarly, extremely low counts were seen in the presence of a normal bleeding time. All observations were made in the early period of the patients' hospital course. The platelet count was found to correlate much more closely with clot retraction, extremely low values being associated with no retraction.

In an attempt to identify possible changes in platelet properties during hemorrhage the mobility of platelets in an electrical field was observed in 10 instances. Nine of these showed values within the normal limits and in the one in which a slight deviation from the normal was found, there appeared to be no correlation with other clinical or laboratory studies. Platelet stickiness studies in 20 patients revealed marked day-to-day variability which defied analysis.

DISCUSSION

The reports concerning the effects of hemorrhage on the platelet count are variable. While Richardson[7] noted a rise in platelet count in rabbits

reaching a maximum four days after bleeding, Drinker and Drinker[8] found no change in dogs in more acute experiments with rapid progressive hemorrhage. Morse[9] described a diminution in platelet levels on the first day following post-partum hemorrhage with a gradual rise reaching a maximum on the thirteenth to eighteenth day. On the other hand, Dawborn[10] studied blood donors following 500 ml. venesection and reported no change in the platelets. Since the degree of hemorrhage, duration of study and experimental species differed in the results reported, they cannot be compared to the present studies.

The observations of the platelets in the patients with massive gastrointestinal hemorrhage reported here demonstrate that there is a definite curve which the platelet count follows during and after bleeding. Since patients ill with severe liver disease have a low platelet count in the absence of hemorrhage, the lower level observed in that group may be explained by factors other than the acute blood loss.

The reason for the lowering of the platelet level during and for several days after bleeding is not clear. It was not possible to demonstrate any platelet depressing factor in the autotransfusion experiments, but the conditions of collection and storage of the plasma may have interfered with its activity. That rapid utilization is responsible in part seems likely but unproved. The lack of relation to amount of blood loss suggests that actual loss by hemorrhage is not the primary cause. Transfusion therapy is also not the primary etiology since the decreases, although frequently observed following administration of blood, remained after the transient effect of transfusion described by Stefanini and Chatterjea[11] would have disappeared. Moreover, the decline was noted in several patients before transfusion.

It is important to note that although platelet levels may be low during the acute episode, there is no evidence that this effect is harmful to the patient by allowing continuation of bleeding. In no case did generalized purpura ensue, nor was there any relation between variation in bleeding time and platelet count. Moreover, thrombocytopenia continued at its lowest levels after hemorrhage had ceased. In view of these studies, it should be emphasized that thrombocytopenia found in the presence of an isolated hemorrhage should not be considered as the etiology of that hemorrhage without further evidence of a primary blood dyscrasia.

The mechanism of the later elevation in platelet count could only be postulated. Actually, the production may increase as soon as the bleeding starts, but may not exceed platelet loss and utilization until later in the course. The factor that stimulates platelet production is unknown. Anoxia is unlikely since no such response is seen in secondary polycythemia. That adrenal hormones may play a part is suggested by the fact that an increase in platelet numbers has been demonstrated on administration of pharmacologic doses to patients with idiopathic thrombocytopenia and to normals.[12]

It is concluded from these studies that during gastrointestinal hemorrhage the platelets remain adequate to play their part in hemostasis along with the

vascular system both in normal patients and in those with severe liver disease, even though the blood platelet count may be found at extremely low levels. In no case was there evidence that platelet defects led to continuation of hemorrhage.

SUMMARY

Significant changes were observed in the platelet count during and after massive gastrointestinal bleeding in patients with and without liver disease. There was a lowering of the count during and for several days after bleeding, followed by a definite rise with convalescence. The decrease in platelet count was not related to the amount of blood loss or to the presence or absence of shock. There was no evidence that the occasional marked drop in platelets contributed to a continuation of the hemorrhage.

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THE EFFECTS OF MASSIVE GASTROINTESTINAL HEMORRHAGE ON HEMOSTASIS

II. Coagulation Factors

Thomas C. Chalmers, M.D., Frederick S. Bigelow, M.D., and
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Boston, Mass.

HEMOSTATIC mechanisms were investigated in a group of patients with massive gastrointestinal hemorrhage to observe changes related to acute blood loss. Significant variations in the platelet count in these patients have been described in the preceding paper.[1] The present paper is concerned with studies of other coagulation factors.

METHODS AND MATERIALS

Coagulation time in glass was measured by the method of Pohle and Taylor.[2] The recalcification time of oxalated plasma was observed according to Quick[3] with the modification of adjusting the pH to 7.35 by equilibrating with a 5 per cent CO_2–95 per cent O_2 gas mixture before recalcifying. Labile factor was determined by means of the technique described by Stefanini.[4] One-stage prothrombin levels were measured according to Rosenfield and Tuft,[5] modified two-stage determinations as described by Ware and Seegers.[6] Serum prothrombin concentration was determined in serum separated one hour after clotting and incubated at 37.5° C. for twenty-four hours; as a source of fibrinogen, plasma freed of prothrombin by barium sulfate absorption was used. The thrombin time was estimated in one of two ways. Thrombin (Parke-Davis bovine) was diluted either in imidazole-buffered saline[7] or in a solution of saline and glycerol to which acacia and imidazole buffer were added in the proportions described in the titration mixture described by Ware and Seegers.[6] The strength of either preparation was adjusted to give a normal time, when added to normal plasma, of between fifteen and twenty seconds. Fibrinogen was measured by the method of Van Slyke[8] and by the method of Quick.[9] Fibrinolytic activity was studied by observation of fibrin clots formed by addition of 0.2 ml. each of 0.02M $CaCl_2$, Difco thromboplastin, and oxalated plasma to 5 ml. of imidazole-buffered saline (pH 7.25), and by observation of whole blood clots. Lysis within twenty-four hours at 37.5° C. was considered abnormal. Fibrinolysis was also measured according to the quantitative method described by Shulman and Tagnon[10] using bovine fibrin as a substrate. The fibrinogen was prepared according to Ware and Seegers[11] and tagged by a modification of the technique of Shulman and Tagnon. The patient's plasma was incubated for sixty minutes at 37.5° C. with tagged fibrinogen, saline, and bovine thrombin. The amount of the clot lysed was calculated by comparing the count of the supernatant with suitable controls.

Clinical data on the patients studied were presented in the previous paper.[1] For purposes of analysis they have been divided into two groups, 45 patients with no evidence of liver disease and 27 with cirrhosis.

RESULTS

A survey of the results of the various tests is presented in Table I.

From the Thorndike Memorial Laboratory, II and IV Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

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TABLE I. SUMMARY OF THE RESULTS OF COAGULATION TESTS IN PATIENTS WITH NO KNOWN LIVER DISEASE AND IN PATIENTS WITH CIRRHOSIS

COAGULATION TEST	PATIENTS WITH NO KNOWN LIVER DISEASE		PATIENTS WITH CIRRHOSIS	
	NUMBER OF EPISODES STUDIED	PER CENT IN WHICH TESTS WERE ABNORMAL ONE OR MORE TIMES†	NUMBER OF EPISODES STUDIED	PER CENT IN WHICH TESTS WERE ABNORMAL ONE OR MORE TIMES†
A. Tests of complete clotting cycle*				
1. Clotting time	5	0	18	0
2. Recalcification time	26	8	19	37
B. Tests for factors contributing to thrombin formation				
1. Labile factor time	17	19	20	85
2. Modified one-stage prothrombin concentration	35	31	34	85
3. Modified two-stage prothrombin concentration	16	38		
C. Tests for factors contributing to fibrin deposition				
1. Thrombin time	28	11	20	55
2. Fibrinogen level	9	0		
3. Fibrinolysis	25	0	32	12

*Modified by effects of glass.
†This includes only those in which value is diminished, not increased.

Coagulation Time and Recalcification Time.—Whole blood clotting time in glass was not prolonged in any case. A total of 42 determinations were made in patients with and without cirrhosis at varying periods during their hospital course. The patients studied included those with all degrees of blood loss.

Recalcification time was observed 66 times during the hospital course of 26 patients without evidence of liver disease. Two values exceeded the upper limit of normal by five seconds and seven seconds, and in each patient other values were found to be within normal limits. In three patients abnormally rapid times were found at some time during their hospital course. In those patients in whom abnormally high or low values were found, no correlation could be made with their clinical state. In only one was there evidence of similar variation in other laboratory tests. This patient had abnormally high prothrombin values at a time when the recalcification time was short.

In the patients with severe liver disease, prolonged recalcification times were noted at some time during seven of 19 episodes. It was not, however, possible to correlate the abnormalities with the patients' clinical condition. Various other defects in the early or late stages of coagulation were noted in all seven of these patients.

Labile Factor.—Only three of 17 patients without liver disease had any evidence of diminution in labile factor. These three all entered with hematocrits below 20 and two of them had evidence of shock. On the other hand, during 17 of 20 episodes of gastrointestinal hemorrhage in patients with severe liver disease, the labile factor was decreased.

Prothrombin.—The prothrombin determinations on blood from patients without liver disease, as measured by a one-stage method with a diluent of prothrombin-free plasma, showed significant variations from the normal. In

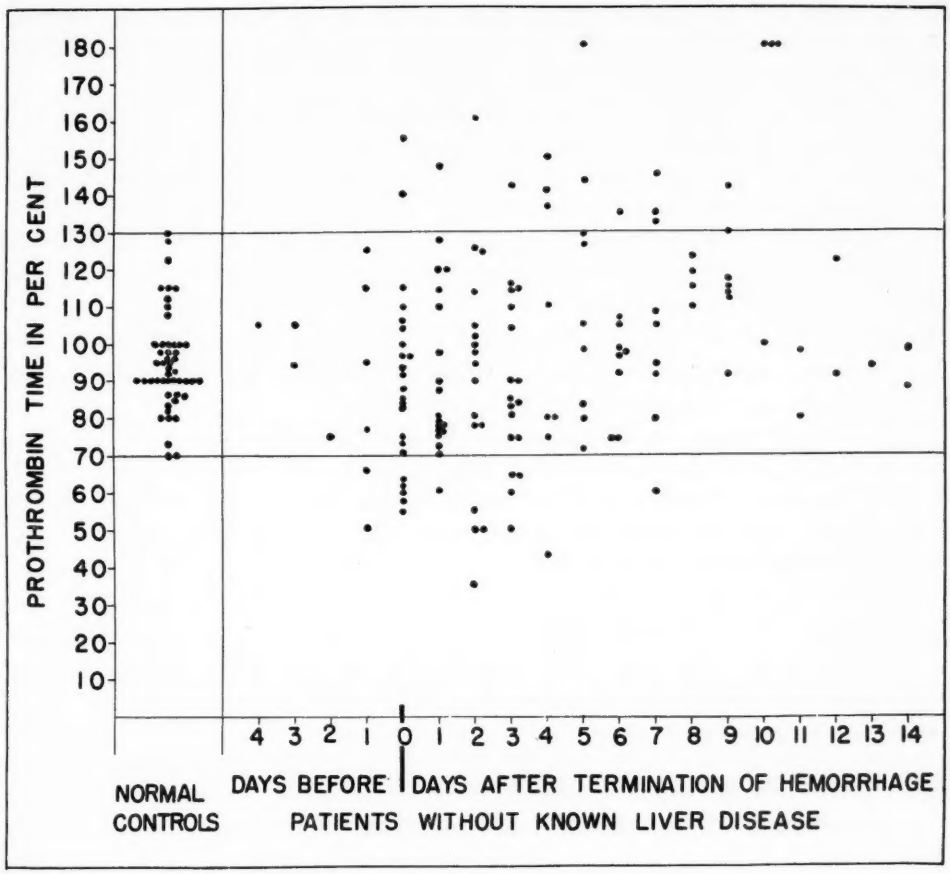

Fig. 1.—The prothrombin concentrations of patients without known liver disease are charted according to the cessation of hemorrhage.

11 patients, 17 values were found below the lower limit of normal. In all but two, later results were within normal limits. When the values were charted according to the time in days after the onset of hemorrhage, low values were noted until the twelfth day. However, when they were plotted in relation to the day of probable cessation of bleeding, all low values except one were found during, or within four days after, active bleeding (Fig. 1). The significant predominance of low values in the patients with admission hematocrits below 25 may be seen in Table II.

Prothrombin concentration was increased 18 times in 14 patients. Only three of the eighteen occurred before the fifth day after onset of hemorrhage. When the values were related to the cessation of bleeding, the same tendency

TABLE II. PROTHROMBIN VALUES IN PATIENTS WITHOUT EVIDENCE OF LIVER DISEASE IN RELATION TO THE HEMATOCRIT LEVEL. ($X^2 = 7.3$. $P = .01$)

PROTHROMBIN CONCENTRATION	LOWEST HEMATOCRIT LEVEL	
	25 OR OVER	LESS THAN 25
Normal throughout	21	3
Abnormal on one or more determinations	4	7

was present for the higher values to occur in the later part of the hospital course. There was a suggestive correlation between the increase in prothrombin values and the cessation of hemorrhage.

In those patients who received transfusions within a day prior to study, there was no significant variation in prothrombin values from those who had not been transfused, and there was also a lack of correlation with the presence or absence of shock.

In sixteen patients without liver disease, prothrombin was followed by the two-stage technique. The results were similar to those observed by the one-stage procedure. Six patients had one or more determinations below normal limits; all six, however, showed return to normal levels at a later date in their hospital course. Seven had elevated values, these being noted after the third day of hemorrhage in six of the seven.

The prothrombin values in the patients with cirrhosis were usually low. Fig. 2 depicts prothrombin levels in these patients with reference to the cessation of bleeding. There was a tendency for the values to rise into the normal range after the cessation of hemorrhage, but many values remained low throughout the observation period.

Prothrombin consumption as measured by serum prothrombin was studied in eight patients with severe liver disease and was found to be normal in all.

Fibrinogen.—Fibrinogen was observed during the course of nine patients with gastrointestinal hemorrhage. Those who were followed after the cessation of bleeding included seven patients with little or no evidence of liver disease. All values were within the normal range, but the curves showed a tendency to rise after bleeding had stopped.

Thrombin Time.—Determinations were made during and after cessation of gastrointestinal hemorrhage in 28 patients without evidence of liver disease. In only three of these was a prolonged thrombin time observed. In two, later determinations were normal, and surgical intervention in the third precluded later studies.

Eleven of 20 patients with cirrhosis had prolonged thrombin times during and after hemorrhage. In these patients no correlation could be found between this and the presence of active bleeding, shock, degree of blood loss, number of transfusions given, fatality, or the need for emergency surgery.

Fibrinolysis.—Complete lysis of the fibrin clot was seldom noted in this series, and when it was found it was transitory and without correlation with the clinical state of the patient.

By using fibrinogen as a substrate an attempt was made to quantitate fibrinolytic activity during five episodes of gastrointestinal hemorrhage. During two episodes in patients with liver disease, there was found on entry increased activity which subsided on further determinations. At no time was complete lysis seen. Both these patients were in shock. During two other episodes in patients with liver disease, no striking changes were found. In one of these, no activity was seen, and in the other, the amount of clot lysis varied from 16 to 8 per cent in specimens taken from the first to the thirty-fourth day. One patient without liver disease was followed and the tests revealed only minimal fibrinolytic activity during the course of the disease.

To investigate further the mechanisms involved in the occasional appearance of lysis during massive hemorrhage, a dog was bled to death over a four-hour period and blood samples studied at intervals. There was no fibrinolytic activity present at the outset, but later samples caused partial lysis of the clot. The serum was also incubated with bovine fibrinolysin and its ability to inhibit this enzyme observed. Before any increase in fibrinolytic activity was found in the dog blood, there was a diminution in its antifibrinolytic activity.

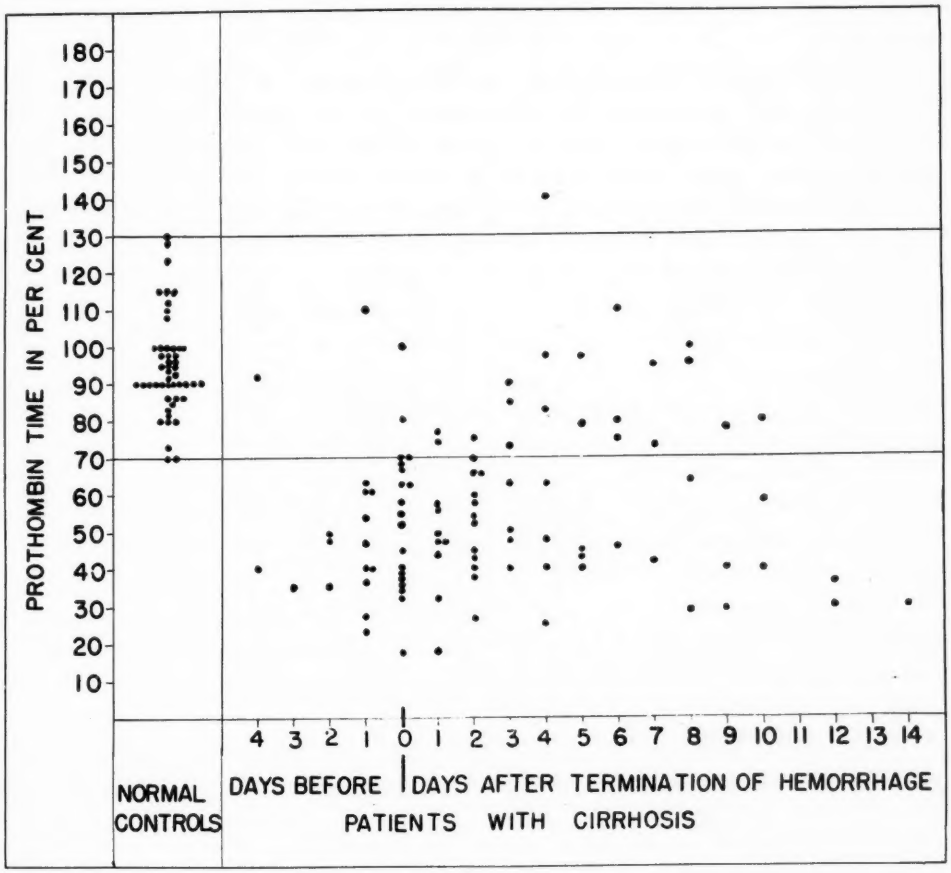

Fig. 2.—The prothrombin concentrations of patients with cirrhosis are charted according to the cessation of hemorrhage.

In order to determine whether fibrinolysins and plasma activated by streptokinase had any effect on other clotting mechanisms before dissolution of the clot could be observed, several in vitro tests were performed. While prothrombin itself was found to be stable, labile factor gradually decreased in the presence of increasing amounts of enzyme activity. Thrombin time became gradually prolonged before enough activity was present to destroy the fibrinogen. Thus, abnormalities in these two tests may also reflect changes in the lysin complex.

Thus while fibrinolysis was never a striking finding in these cases, there were, however, changes observed in labile factor and thrombin times which could have been a reflection of changes in the lysin complex.

DISCUSSION

There have been some reports in the literature which suggest that coagulation disturbances may occur following massive hemorrhage. Frank and others[12, 13] demonstrated decreased prothrombin activity in dogs with hemorrhagic shock and also noted a decreased ability to regenerate fibrinogen in these animals. Fibrinolysis has also been described as an accompaniment of shock.[14, 15]

The differences between patients with cirrhosis and those without liver disease are similar to those described previously.[16] Besides low prothrombin values, there were many patients with prolonged recalcification time, decreased labile factor, and prolonged thrombin time in the group with severe liver disease. The prolonged recalcification time in the patients with cirrhosis could not be attributed to any single defect. That prothrombin conversion was adequate is evidenced by normal prothrombin consumption. Since varying degrees of defects in other factors were noted, the results of this test might be considered a summation of other abnormalities.

The lowered prothrombin levels in the patients without liver disease appeared to be more closely related to the amount of blood lost than to any other clinical criterion. Liver function tests at this time were usually normal and it is unlikely that the defect can be attributed to anemic anoxia of the liver. Limited production plus increased utilization in this situation may explain the decreased prothrombin. That the same tendency is present in patients with cirrhosis is not surprising.

Fibrin formation as measured by thrombin time was abnormal in many cirrhotics. This could not be correlated with any clinical parameter related to hemorrhage, however, and was perhaps more closely related to the underlying liver disease.

Although fibrinolysis has been reported in shock, the finding was extremely uncommon in this series of patients. Since hemorrhage severe and prolonged enough to result in generalized bleeding at the time of operation was not encountered in this series, fibrinolysis may still be important in such a situation. The quantitative studies demonstrate that there may be some increase in fibrinolytic activity in the blood of patients with gastrointestinal hemorrhage without a rise sufficient to cause total lysis of the clot in vitro. Such activity had no obvious detrimental effect on the clinical course. The observations in the dog suggest that a decreased antifibrinolytic activity plays a part in this elevated fibrinolysis occasionally seen during bleeding.

From the results of these studies, it is evident that hemostasis is maintained adequately following massive gastrointestinal hemorrhage even in the presence of severe liver disease. Decreased prothrombin levels did not lead to further bleeding. The tendency for increased prothrombin values and

increased fibrinogen levels later in the course may be considered compensatory mechanisms, but hypercoagulability was not a striking finding in this group of patients either by laboratory or clinical findings.

The observations of coagulation are similar to those of platelets in the preceding paper in that there was no evidence that any defect led to the continuance of bleeding. These tests fail to reveal any need for special types of replacement therapy other than the use of blood transfusions to maintain adequate hematocrit levels. They do not, however, exclude the possibility that more striking coagulation defects may appear in patients with more massive hemorrhage, treated less promptly.

SUMMARY

Abnormalities in over-all tests of clotting and in those designed to measure factors contributing to thrombin formation and fibrin disposition have been found in patients with gastrointestinal hemorrhage.

They were much more frequently encountered in patients with cirrhosis of the liver.

They were probably of little or no clinical significance in this group of patients but indicate the possibility of more important changes occurring in patients with more severe hemorrhage treated less promptly.

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THE EFFECTS OF WASHING AND OF SERUM ALBUMIN UPON ERYTHROCYTES

I. Upon Morphology as Seen With Phase Microscopy and With Polarized Light

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Furchgott and Ponder[1] found that saline-washed erythrocytes decrease in diameter and become sphered when allowed to film between closely approximated glass surfaces, and that this phenomenon can be prevented by addition of small amounts of serum, or of serum albumin, to the saline suspensions. These phenomena have been re-examined recently in the course of inquiry into the actions of crystalline serum albumin upon hematopoietic tissues.[4, 5] The findings serve to affirm those of Furchgott and Ponder. The tools and proportions of albumin used for these investigations differ from those employed by them, however, with the result that additional phenomena of erythrocytes have also been brought to light in relation to their response to serum albumin. The contributions of this series will deal largely with these additional data.

METHODS

The donors were the same women as those used in establishment of the method employed for the measurement of erythrocytic diameters.[6] The blood of M.A.G., who is of Italian extraction, contains numerous ovalocytes which had to be omitted in the determinations of mean diameter. All three donors were found normal according to the standard medical examination given to the personnel of this Institute. Defibrinated blood was used for all determinations except the control films of whole blood. The blood was obtained by deep puncture of a finger and allowed to run down the sides of a centrifuge tube along which most of the clot formed and stuck. The serum was removed by spinning and saved at 4° C. for the determinations which demanded donor's serum. A portion of the cells was stored at the same temperature for the determinations upon unwashed, defibrinated cells. The remainder of the cells was washed four times with Locke's solution and stored in the last bath at 4° C. Precautions were taken against inclusion of clots in each case.

Ten per cent solutions of crystalline bovine serum albumin (Armour) in Locke's solution were used as stock for preparation of all weaker solutions of albumin. The dilutions were made with Locke's solution. The Locke's solution used for the present studies (lot 1) had a freezing point of 0.69 and a pH of 6.35.

Mixture of any given batch of cells with the specific medium desired was attained by placing 0.02 ml. of the medium upon a slide and mixing into it a loopful of the cells under study. The volume was about ninety times that of cells. Drops of the mixture were immediately placed upon cover glasses, inverted onto slides, and rimmed with

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petrolatum to prevent evaporation. The glassware was cleaned, and the measurements of diameters were carried out under phase microscopy as described elsewhere.[6] All preparations were let stand at room temperature for ten minutes before measurements were begun, in order to assure that the cells had attained equilibrium. Films of fresh drops of whole blood, and of the cells stored in serum or in Locke's solution, were rimmed and examined in the same manner.

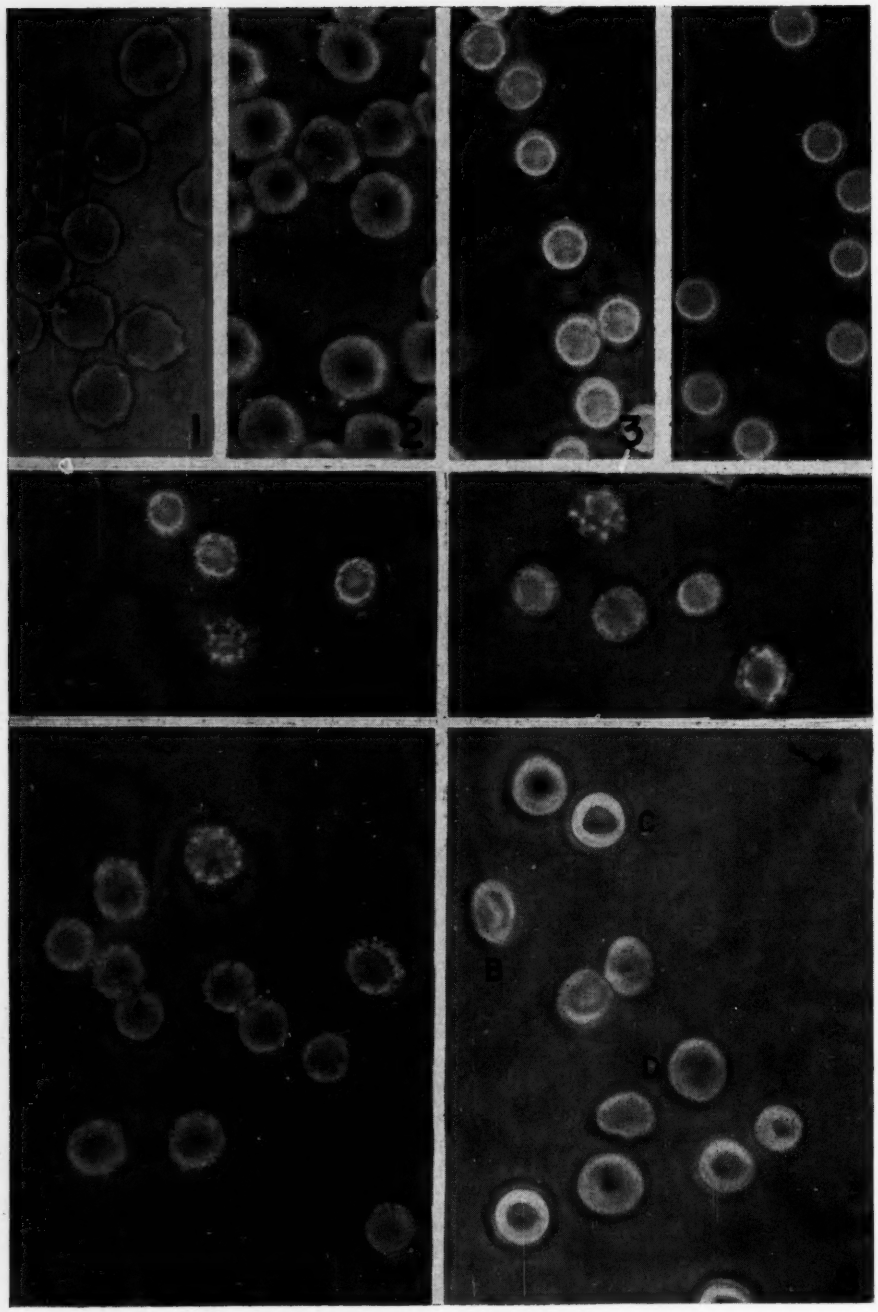
Examination of the same films by polarized light was attained by means of plates of polaroid inserted into an ordinary "research" microscope by the manufacturer. Intense Koeler illumination was used.

RESULTS

Unwashed Erythrocytes Suspended in Plasma or Serum.—The peripheral aberrations which are characteristic of erythrocytes in unfixed films of blood when examined with standard bright-field illumination of the microscope (Fig. 1) disappear under phase microscopy and the cells appear sharp and clear-cut and easy of measurement[6] (Fig. 2). They appear dark or light against a background of opposite hue, dependent upon which level of phase is employed. Otherwise they appear as with bright-field illumination. They are disc shaped, have paler centers than peripheries, and are in Brownian movement. In addition, internal movements, which are practically undetectable with bright-field illumination, can easily be seen with a phase microscope.[7] The cells retain these two forms of movement for long periods when they are in plasma or serum; they settle slowly to the slide; and they exhibit just the faintest hint of birefringence about the periphery, under the conditions of observation employed (cf. Schmitt, Bear, and Ponder)[3] so long as crenation remains in abeyance (Fig. 11). The diameters vary from cell to cell, but the average is relatively constant from individual to individual (Charts I-III). The mean value for the erythrocytic diameters of each of the three individuals under study is 8.9 microns.[6]

Washed Erythrocytes Suspended in Locke's Solution.—The cells appear completely metamorphosed under these circumstances when viewed between glass by phase microscopy (Figs. 3 and 4). Central cupping disappears and the cells look homogeneous throughout. They appear as dense white plaques with either finely crenated (Fig. 3) or sharp round (Fig. 4) edges. They exhibit diffuse birefringence of low intensity in polarized light (Figs. 10 and 12) in contrast to its absence in the normal cells under the same optical conditions, and more intense birefringence of the edges than that of the edges of normal erythrocytes (Fig. 11). The diameters are markedly decreased compared to the normal (Charts I-III). Brownian movement has coarsened and decreased in frequency, and internal rhythmic movements have ceased. The cells settle rapidly to the slides.

Washed Erythrocytes Resuspended in Serum or in Solutions of Albumin in Locke's Solution.—The cells progress through a series of changes upon resuspension in either medium (Figs. 5-9) which represent the transitional forms from spheres to discs, and vice versa, described in detail by Ponder.[2] These include coarse crenations (Figs. 5-7), fine crenations (Figs. 5 and 6), cup and umbrella-shaped forms (Figs. 8, *B* and 9, *B*), doughnutlike forms (Figs. 8, *C* and 9, *C*), crescents, etc., until the ultimate static state of normal-appearing



Figs. 1-8.

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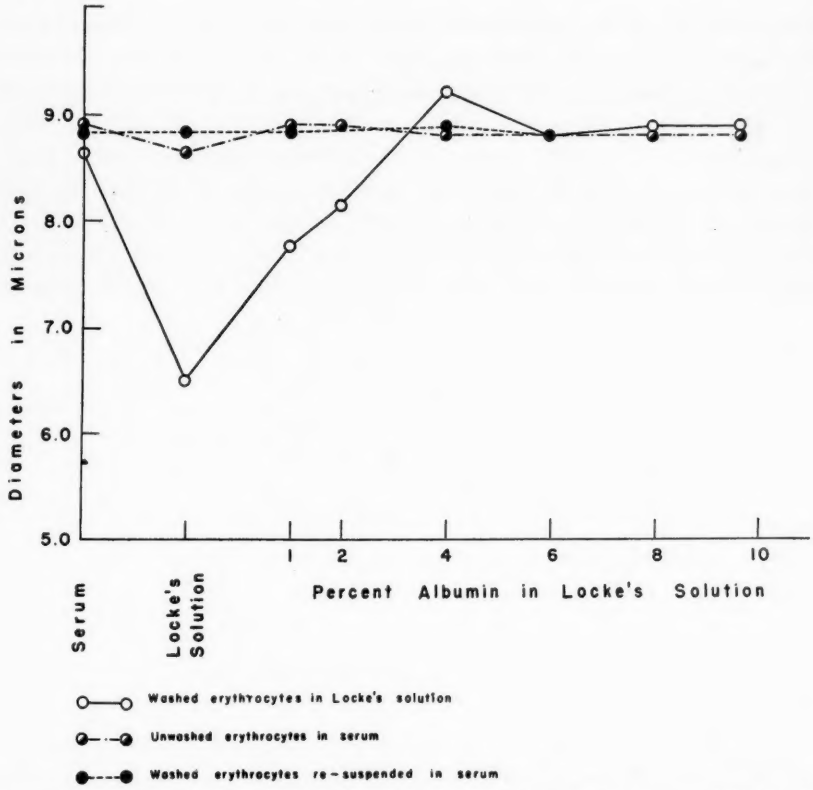


Chart I.—Diameters of erythrocytes (M. A. G.) with and without additions of albumin when suspended in serum, or after washing in Locke's solution, or when resuspended in serum after washing.

Fig. 1.—Normal blood as seen with the ordinary microscope and standard bright-field illumination. The edges of the erythrocytes are surrounded by diffraction bands which make sharp definition impossible. Oil immersion objective (1.4 n.a.) ×15. (×1,350; reduced ⅕.)

Fig. 2.—Same film as Fig. 1 viewed with phase microscopy. The edges of the erythrocytes are sharp and easily defined. Margins and central cupping seem deepened and the cells appear embossed. Rhythmic movements within the erythrocytes showed plainly but were not disturbing photographically. Bright medium-phase oil immersion objective ×15. (×1,455; reduced ⅕.)

Fig. 3.—Erythrocytes after four washings with Locke's solution. They have become almost spherical with shortening of the diameter and loss of central cupping. Delicate crenation remains. The cells are opaque and dense. Internal rhythmic movements have ceased. (cf. Fig. 10.) Bright medium-phase contrast microscopy. (×1,455; reduced ⅕.)

Fig. 4.—Same lot of erythrocytes as for Fig. 3 except that the suspension has been made slightly alkaline. Sphering is complete and the delicate crenations present at the pH of the suspension used for Fig. 3 have disappeared. Otherwise the characteristics are as in Fig. 3. (cf. Fig. 12.) Bright medium-phase contrast microscopy. (×1,455; reduced ⅕.)

Fig. 5.—Same lot of erythrocytes as for Fig. 3 after addition of a few drops of crystalline bovine serum albumin in Locke's solution. The erythrocytes show evidence of beginning revision to normal status. Coarse crenation has appeared but the cells are still homogeneous, dense, and without central cupping. Bright medium-phase contrast microscopy. (×1,455; reduced ⅕.)

Fig. 6.—Same lot of erythrocytes as for Fig. 5 with addition of slightly more albumin. Some of the cells are still sphered while others show changes that represent stages in reversion toward the normal status, but translucence and central cupping are still lacking except suggestively in the cell marked by an arrow. Bright medium-phase contrast microscopy. (×1,455; reduced ⅕.)

Fig. 7.—Same lot of erythrocytes as for Fig. 6 with addition of more albumin. Most of the cells show beginning translucence and cupping with evidences of increasing fluidity in such areas. Bright medium-phase contrast microscopy. (×1,455; reduced ⅕.)

Fig. 8.—Same lot of erythrocytes as for Fig. 7 with addition of more albumin. All of the cells now show evidences of fluidity and two (*D*) have regained normal translucence, central cupping, and disc shape. The other cells represent the mushroom (*B*) and doughnut (C) shaped forms that characterize the end stages in reversion of spheres back to discs. (cf. Fig. 9.) Bright medium-phase contrast microscopy. (×1,455; reduced ⅕.)

cells (Figs. 8, *D* and 9, *D*) is regained. Only partial return to normal appearance and behavior was attained with weak solutions of albumin within the conditions of these experiments (Figs. 5-7). Furchgott and Ponder[1] obtained a return to the normal status with lesser concentrations under the conditions of their experiment. Complete return in the present experiment was attained within the waiting period of ten minutes with serum, and with the higher concentrations of albumin in Locke's solution (Figs. 8, *D* and 9, *D*). Once returned to normal, the cells regained their usual rapid Brownian and internal movements, central cupping and disc shape, and decreased in birefringence.

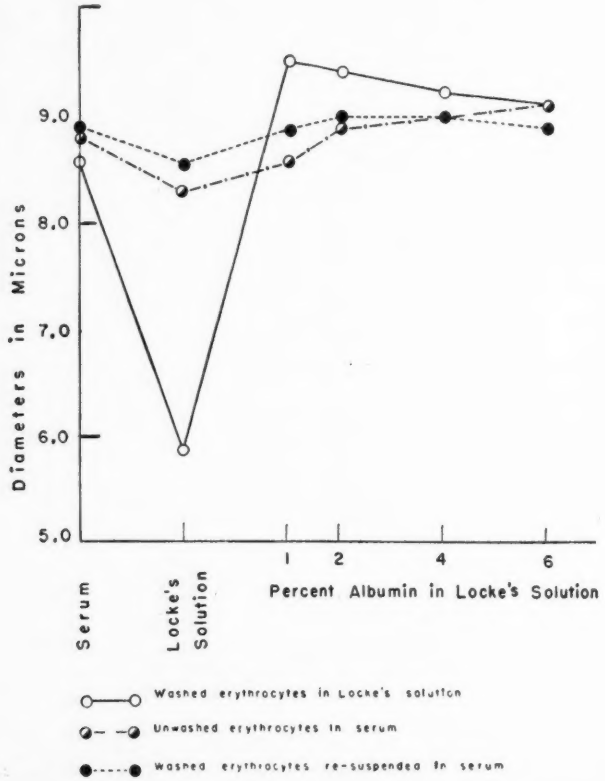


Chart II.—Diameters of erythrocytes (E. H. T.) with and without additions of albumin when suspended in serum, or after washing in Locke's solution, or when resuspended in serum after washing.

At the same time the diameters increased (Charts I-III). Having returned to normal by resuspension of the cells in serum or in the higher concentrations of albumin in Locke's solution, the diameters remained unaffected by increasing quantities of albumin within the limits of these experiments. (Charts I-III). In the case of resuspension in the lesser concentrations of albumin (1.5 to 4.0 per cent), on the other hand, in which the cells acquired the bizarre forms described above, the average diameter was greater than normal. This was probably an artifact occasioned by the selection of cells and inherent in the state of the cells, rather than real. Diameters could be measured only

on cells that were symmetrical, i.e., discs or spheres. The various crenated or bizarre formations between spheres and discs did not lend themselves to measurement. Spheres were excluded from the measurements as not yet under the influence of albumin. Only those erythrocytes were measured, therefore, that had regained the form of discs. The reason why but a limited number of the population of erythrocytes attained the disc stage is unknown.

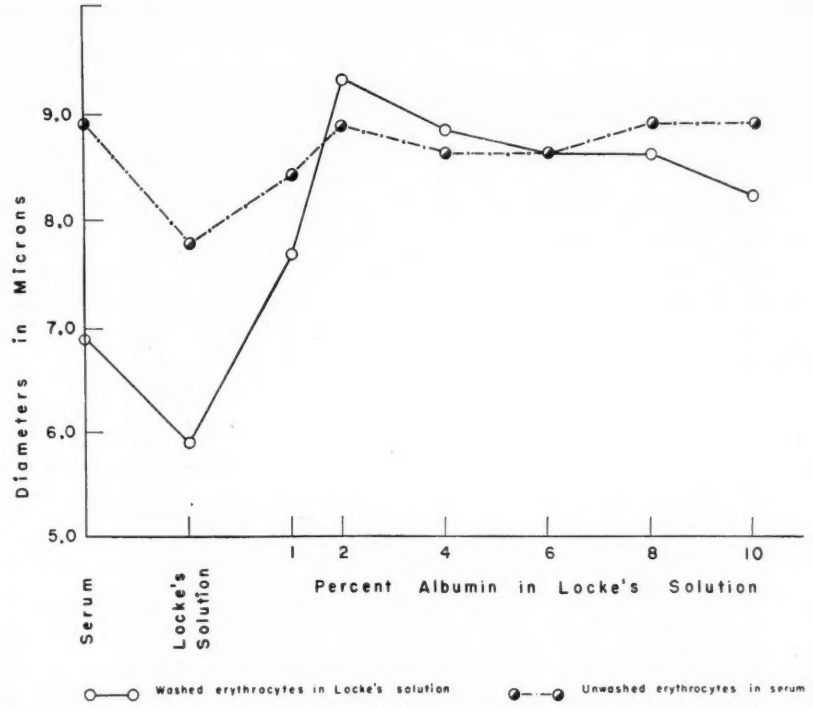


Chart III.—Diameter of erythrocytes (A. F. G.) with and without additions of albumin when suspended in serum or after washing in Locke's solution.

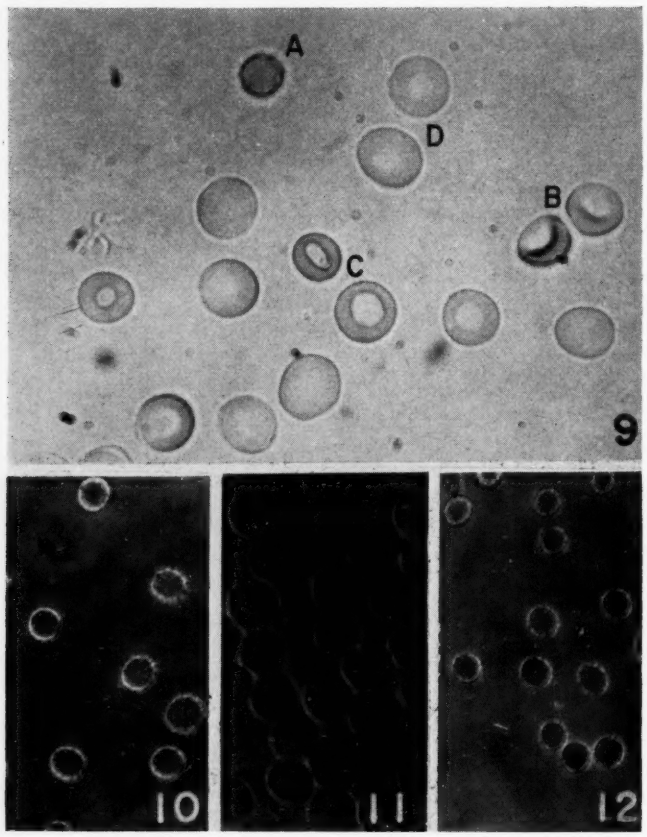
It would seem that albumin may be unequally distributed when in low concentration and that certain cells benefit at the expense of the remainder. Why these should be of larger diameter than normal is not certain, unless the larger (younger) cells of the population have greater powers of attraction than the remainder, and hence are the first to become coated and measurable. When all the cells become reconverted to discs in the higher concentrations of albumin, these large ones would represent only a small part of the mean and weight it less heavily.

In review, then, the foregoing studies corroborate the findings of Furchgott and Ponder to the effect that washed erythrocytes become sphered and decrease in diameter when spread between glass surfaces. They also reveal that the washed cells lose central cupping, become opaque, and increase in birefringence upon development of the sphering phenomenon, and that Brownian movements become slowed and coarsened, and the characteristic internal movements of erythrocytes in serum are lost. Addition of serum or

of solutions of albumin is followed by restoration of the discoid shape and by return to normal of the diameters, movements, and other morphologic characteristics.

DISCUSSION

The increased speed with which the washed cells settle to the slide, their homogeneity, opaqueness, optical denseness, and faint diffuse birefringence all indicate conversion of their contents from a fluid to a more congealed



Figs. 9-12.

Fig. 9.—Same lot of erythrocytes as for Fig. 5 after addition of 1.5 per cent albumin. Viewed with an ordinary microscope and standard bright-field illumination for comparison with similar cells shown by phase microscopy in Fig. 8. (Equivalent lettering used for both figures.) The erythrocytes had assumed various transitional forms between spheres and discs. Some were still sphered (A), some mushroom or umbrella shaped (B), some doughnut shaped with thick rims and apparently nonhemoglobin containing centers (*C*), and the greater proportion normal (D). (×1,260; reduced ⅕.)

Fig. 10.—Erythrocytes after four washings with Locke's solution. Viewed with polarized light. They were diffusely but softly birefringent throughout. Their delicately crenated edges were brilliantly polarized. (cf. Fig. 3.) Oil immersion ×10. (×900; reduced ⅕.)

Fig. 11.—Film of normal blood viewed with polarized light for comparison with the erythrocytes shown in Figs. 10 and 12. The edges of the cells are very dimly birefringent. Birefringence is otherwise not evident under the conditions of polarization employed. (×900; reduced ⅕.)

Fig. 12.—Same lot of erythrocytes as for Fig. 8 except that the suspension was made sufficiently alkaline to complete spheridity. The same statements apply to the completely sphered cells with smooth edges as to those shown in Fig. 10. (cf. Fig. 4.) Polarized light. (×900; reduced ⅕.)

status. They suggest the possibility that the contents have changed into a gel or paracrystalline state, concomitant with the removal of serum from their surfaces. Replacement of albumin to the medium results in reappearance of the signs of fluid status.

Furchgott and Ponder[1] showed conclusively that it is their coating of serum albumin which protects erythrocytes from sphering when placed between closely approximated glass surfaces. Ponder[2] has shown that this coats the ultrastructure to a depth of 50 A. The reason for the protection afforded against sphering is not clear. Ponder postulates a deep penetration of the ultrastructure by albumin with reorientation of molecules which lend stability to the ultrastructure. He found the protective action of albumin to function in infinitely smaller amounts than those used in the present experiments and without alterations of the volumes of erythrocytes.

The present studies show that albumin protects erythrocytes not only against sphering but also against gelation or solidification. In this instance, also, the findings do not permit conclusion as to why removal of serum albumin should elicit conversion of the cytologic contents to a congealed state or, vice versa, why addition of albumin to washed erythrocytes should return their contents to fluid status. It would seem either (1) that water must be subtracted from the contents of the cells, or (2) that other molecules must be admitted when the contents pass into the gelid state (and, vice versa, water returned to the cells along with the serum albumin, or other molecules removed when the contents return to fluid status) or, finally, (3) that reorientation of molecules with the formation of compounds of lesser solubility, or of colloidal aggregates, must take place to cause the contents to become gelid upon withdrawal of albumin, and vice versa.

CONCLUSIONS

Saline washed erythrocytes become opaque, homogeneous, and birefringent when admitted between closely approximated glass surfaces. They also lose their normal internal movements and become slowed in Brownian movement. These changes seem to indicate conversion of their contents to a more gelid status. They likewise become sphered and decrease in diameter.[1]

Addition of serum, or of crystalline serum albumin, to the suspension of washed cells is followed by return of normal morphology and movements. The contents appear to have returned to fluid status.

The reason for the effects of albumin upon erythrocytes is not determined. It is discussed as probably due either to reorientation of molecules with formation of more soluble groupings, or to deep penetration of albumin within the ultrastructure concomitant with imbibition of water and increased fluidity of the contents.

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THE EFFECTS OF WASHING AND OF SERUM ALBUMIN UPON ERYTHROCYTES

II. Upon the Internal Movements of Erythrocytes

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MOVEMENTS within erythrocytes were first described by Browicz in 1890.[2, 3] He observed them in anemic blood and thought them due to the movements of large molecules within the cells but did not elaborate further. The phenomenon was also known to Cabot.[4] It was not reported upon again until 1936 when it was described by Forkner, Zia, and Teng[5] without awareness of the earlier reports. They found it present in the blood of various species, anemic or otherwise. They, too, consider it due to the movements, which seemed rhythmic to them, of large molecules within the cells. The movements were described still again in 1950 by Pulvertaft[9] after the advent of phase microscopy and without awareness, apparently, of the contribution by Forkner, and co-workers. He, likewise, considers them due to the movements of large molecules within the erythrocytes. He found that they are obliterated by exposure of the cells to sodium fluoride, but not modified by exposure to carbon monoxide or sodium cyanide, and therefore not due to the oxidation and reduction phenomena of hemoglobin. Blowers, Clarkson, and Maizels[1] corroborated the findings of Pulvertaft and added the information that the movements are also obliterated by various sphering agents and by monoiodoacetic acid as well as by sodium fluoride. Since the latter chemicals inhibit enzymes concerned with anaerobic glycolysis, these authors suggest that the rhythmic movements are related to the shape of the cells and due to the movement of molecules concerned with their glycolytic metabolism. Weis-Fogh[13] draws the same conclusion in abstract. Parpart, Hoffman, and Green[7] believe them due specifically to the discoid shape of erythrocytes.

METHODS

The present studies of the rhythmic movements of erythrocytes were carried out at the same time that measurements and morphologic studies were being made upon the same cells under various conditions.[12] Unfixed erythrocytes from three normal women were examined between slide and cover slip under the following conditions: (1) suspended in serum or plasma before having been washed in Locke's solution; (2) after thorough washing in Locke's solution; (3) after resuspension of the washed cells in the donor's serum; and (4) after addition of various concentrations of serum albumin to the suspension of washed cells. A phase microscope equipped with a bright medium-phase contrast oil immersion objective and 15× oculars was used for these studies.

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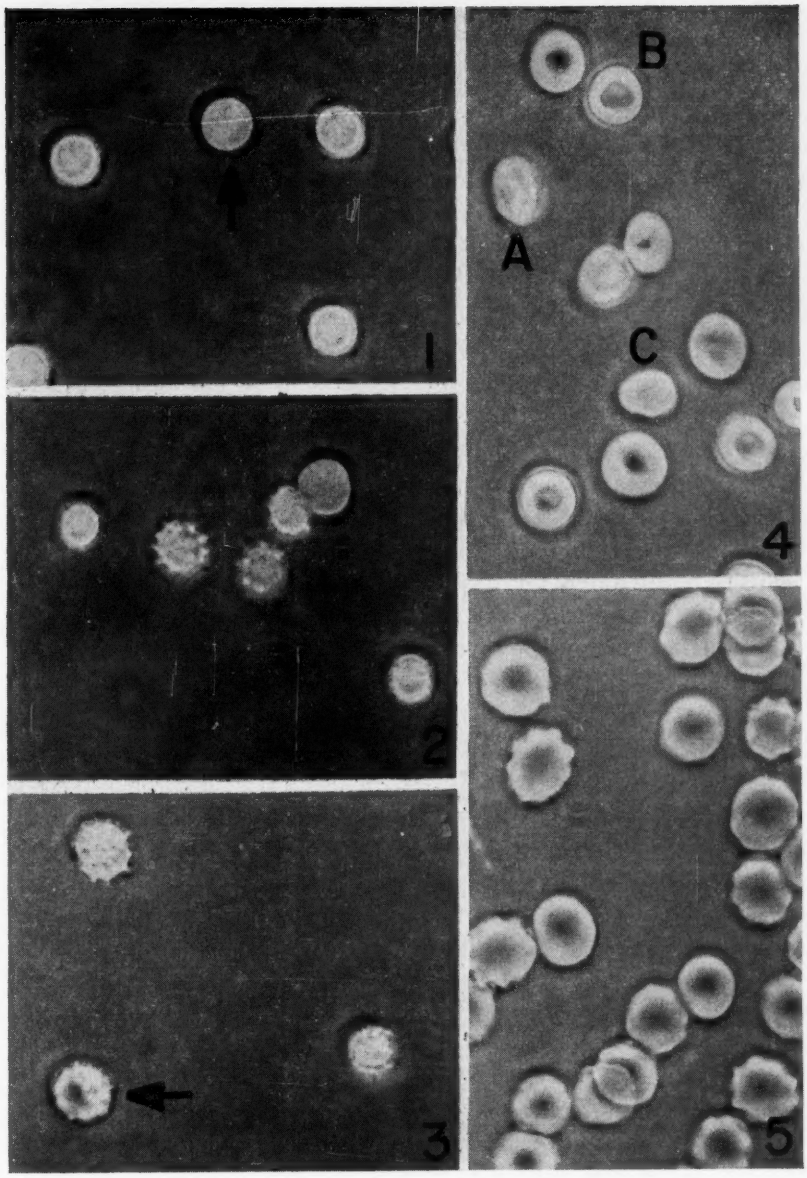
RESULTS

The movements within erythrocytes are clearly discernible in unfixed films of whole blood or of centrifuged sediments from clotted blood when examined by any form of phase contrast microscopy, but they show best under bright, medium-phase contrast microscopy. They are represented by a constant shimmer, or fluctuation between brightness and dimness, beneath the surface of the cells. They appear most clearly at the centers of the erythrocytes where they seem to swirl either circumferentially or radially. They cannot be seen at the edges and they do not occur at crenated points of the surface, although still present in the translucent areas within the capsule. They reminded Forkner, and co-workers,[5] of the appearance of an aurora borealis. They barely show in cells on edge except as a quivering effect at the area of the concavity. They show plainly in the top and bottom cells of a rouleaux viewed from the surface, and as a generalized shimmer along the entire surface of the rouleaux where the cells are closely overlapped. Unlike the Brownian movements of definable particles, these movements are unaccompanied by changes in the cells' position in space and do not interfere with photography of cells by time exposure. They seem more rapid, rhythmic, and systematic than the Brownian movements of the cells themselves or of the chylomicrons in the plasma around them, and without the aberrations of focus which are characteristic of definable particles in Brownian movement. They are more like the shimmer which fills the background of plasma between the visibly oscillating chylomicrons. They continue, and seem unabated in frequency, regardless of whether the cells themselves are in free movement in the plasma or have settled to quiescence against the slide. The term "flicker" has been applied to these movements, but their significance seems too little understood at the present time to justify the connotations of that term.

The internal rhythmic movements become obliterated when saline washed erythrocytes are allowed to spread between glass surfaces. This is true whether the cells settle to the slide or remain suspended in Brownian movement. The internal movements return if the cells are resuspended in serum before spreading, or if sufficient serum albumin is added to their suspension in Locke's solution. They are absent from the early intermediate stages of morphologic response to albumin, which are characterized by fine crenation of cells that are otherwise still sphered (Fig. 1), and also from forms with coarse crenation but still lacking areas of translucence (Fig. 2). The movements become visible, however, in the areas of translucence, from the appearance of the first areas to the stage of completely recovered discs (Figs. 4, *C* and 5). They occur in the small translucent spots of coarsely crenated forms (Fig. 3), in the folded areas of the cup or umbrella shaped forms (Fig. 4, *A*), and in the narrow rims of the doughnut-shaped forms (Fig. 4, *B*).

DISCUSSION

It has been shown that saline-washed erythrocytes assume characteristics, when admitted between closely approximated glass surfaces, which are con-



Figs. 1-5.—All photographs are of unfixed erythrocytes from the same human. All were taken with bright, medium-phase contrast microscopy. All are at the same magnification, ×1,455. Reduced ⅕.)

Fig. 1.—Erythrocytes between glass after four washings in Locke's solution. The cell at the arrow is completely sphered. The others are almost sphered but still show fine crenations. All are homogeneously dense and without any areas of translucence. Internal movements had ceased in all.

Fig. 2.—Same suspension of washed erythrocytes as for Fig. 1 after addition of a small amount of serum albumin. One cell is still unaffected. The others show the coarse crenation of the first stages of reaction to albumin but are still dense and without areas of translucence and were lacking in internal movements.

Fig. 3.—Same cells as for Fig. 1 with addition of slightly more albumin than for Fig. 2. The cell at the arrow has a small area of translucence in which internal movements were evident. Otherwise the same statements apply as for Fig. 2.

Fig. 4.—Same suspension of cells as for Fig. 1 after addition of more albumin than for Fig. 3. The cells show transitional stages toward the normal. Some are mushroom shaped (*A*), some doughnut shaped (*B*), and a few have recovered normal appearance (*C*). Internal movements were visible in all. They were most prominent at the flange of the mushroom in cells of the type shown at *A*, at the inner surface of the rim bordering on the hollow in cells of the type shown at *B*, and over the area of the concavity of recovered cells such as those shown at *C*.

Fig. 5.—Film of normal human blood for comparison with Fig. 4, *C*. Internal movements were evident in all cells. These were most prominent at the periphery of the concavity and extended both circumferentially and radially.

sidered as indicative of a gelid or paracrystalline state of their contents[12]; and that resuspension of the washed cells in serum, or additions of serum albumin to their suspension in saline solution, is followed by reversion to normal appearance and seeming fluidity. The internal movements of erythrocytes, then, cease under conditions that favor gelling of their contents and return under those that induce fluidity. The fact that sickled cells, in which the hemoglobin occurs in crystallized form,[8, 10] lack these movements[9] is in agreement with this finding. The movements depend, apparently, upon a fluid status of the contents. This status, in turn, has been shown to be dependent, in normal cells, upon the presence of serum or of serum albumin in the environment.

It is true that the rhythmic movements occur or cease in the present experiments under the same conditions that cause the erythrocytes to become discoid or sphered, respectively, and that their dependence upon shape only must therefore be considered. The experimental data, in toto, however, seem to indicate that the modifications of both shape and movement should rightly be regarded as dependent upon a common factor, that is, change in the physical status of the erythrocytic contents, rather than as directly interdependent.

The cessation or return of the internal movements under the conditions of gelling or of fluidity, respectively, lends support to the concept that they are related to the movements of large molecules within the cells, since it is to be assumed that movements of molecules of colloidal dimensions would decrease or increase, respectively, parallel with gelling or fluidity. This would be true whether the movements represent simply the aggregate effect from the Brownian movements of innumerable submicroscopic colloids of all sorts within the enclosed area of the erythrocytes, or whether only specific molecules are involved, i.e., hemoglobin, glycolytic enzymes, or other specific colloids. The present observations lend support only to the former more generalized concept. Under this concept, and unlike the situation when erythrocytes are exposed to specific toxins,[1, 9] the effects upon their internal movements should be, and are, reversible. The rhythmic movements, and apparently molecular ones, can be brought back to normal by reversal of the physical status of the contents of washed erythrocytes, and this can be achieved merely by replacement of serum albumin to their medium.

CONCLUSIONS

The internal movements of unfixed erythrocytes are obliterated when the cells are spread between glass after they have been washed in Locke's solution. The movements return if the cells are resuspended in serum before spreading, or if serum albumin is added to the saline suspension.

The morphologic changes that take place in washed erythrocytes when spread between glass are regarded as due to conversion of their contents to a more gelid status than normal, and the changes following exposure to serum albumin as due to reversion to a fluid status.[12]

Correlation of these findings leads to the following deductions: (a) Maintenance of the normal internal movements of erythrocytes requires that their contents be fluid. (b) The presence of serum or of serum albumin in the environment of erythrocytes is important to retention of a fluid status of their contents and, therefore, to maintenance of their internal movements.

The findings are discussed as suggestive that the internal movements of erythrocytes represent the composite effect of the Brownian movements of innumerable submicroscopic colloids within the confines of the capsule.

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BLOOD GROUP MUCOIDS: THEIR DISTRIBUTION AND GROWTH-PROMOTING PROPERTIES FOR LACTOBACILLUS BIFIDUS VAR. PENN

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THE growth factor for *Lactobacillus bifidus* var. *Penn* has been shown to consist of N-containing oligo- and polysaccharides having as building stones the same simple sugars as blood group mucoids.[1, 2] It therefore seemed appropriate to determine whether other relations exist between the growth factor and the blood group mucoids. In this study the properties of these substances have been compared; their distribution throughout the human body has been investigated. Their microbiologic and serologic activities following purification have been examined.

EXPERIMENTAL

Preparation of Substrates.—

Secretions:† Human secretions from physiologic and pathologic sources have been tested, as shown in Tables I and II. All specimens were heated in a boiling water bath for ten minutes, in order to destroy enzymes which might bring about false agglutination in the agglutination inhibition test. All secretions were free of blood contamination.

Colostrum was difficult to test, because of the presence of numerous solid particles even after centrifugation at 3,000 r.p.m. Bile was toxic for *L. bifidus* and lysed erythrocytes at the low titers of the agglutination test. Upon dialysis the toxicity decreased. An actively growth-promoting mucoid material which did not cause hemolysis remained after the repeated extraction of lyophilized bile with 95 per cent ethanol. The studies reported here were made with almost pure gall bladder mucus found in that form of chronic cholecystitis referred to as hydrops of the gall bladder. The cervical mucus was obtained in most instances from autopsies performed within three to eight hours after death. Only clean samples were collected. In one instance a large Nabothian cyst was obtained from a surgical specimen. The colon mucus examined came from a large cyst, which lay behind the transverse colon and appeared to the surgeon as a duplication of colon. Histologically, it had all the characteristics of colon. The bronchial mucus was obtained from tracheotomized patients or autopsies.

Tests on whole saliva are reported here. The supernatant from centrifuged saliva gives essentially the same values. Meconium was shaken with distilled water, centrifuged, and the supernatant fluid thoroughly dialyzed. The pleural, pericardial, peritoneal, spinal, and synovial fluids were obtained from living persons and from autopsies. They were also thoroughly dialyzed before testing. Sweat from different persons was collected on pieces of gauze, extracted with distilled water, and concentrated in vacuo.

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TABLE I. DISTRIBUTION OF BIFIDUS FACTOR AND BLOOD GROUP MUCOIDS IN HUMAN SECRETIONS

SECRETION*	NO. TESTED	BIFIDUS FACTOR (MG./ UNIT)	RECIPROCAL OF INHIBITION TITER			NO. WITHOUT A B H ACTIVITY
			A	B	H†	
Colostrum	10	1-3	20		40	1
Milk	10	2-8	20-160	20-160	40-2,560	0
Gall bladder mucus	2	1	25,600		400	0
Pseudomucinous ovarian cyst fluid	8	1-3	204,800-6,400,000	3,200,000	6,400-512,000	0
Cervix uteri mucus	8	1.5-2	800	800	1,600	3
Meconium	30	0.5-3	100-3,200,000	100-3,200,000	100-1,600,000	6‡
Bronchial mucus	4	1.5-3	800	800	800	0
Nasal mucus	1	3			400	0
Saliva	20	2-2.5	12,800-3,200,000	12,800-102,400	800-102,400	5§
"Colon" mucus	1	3	409,600		25,600	0
Mucocele of appendix	2	3.5	200			1
Amniotic fluid	5	ca.10	800-1,600	800-12,800	800-1,600	2

*If the material was not fluid it was prepared as a suspension in water. Colostrum and milk were skimmed.

†Eel or rabbit antiserum.

‡Four of these samples were tested for Le^a factor. All were positive, the titer being >12,800 in 3 cases, <12,800 in one case.

§These samples were Le^a positive: >12,800 in 4 cases, <12,800 in one case.

Extracts of Human Organs.—Normal and pathologic tissues were examined. The starting material obtained four to twelve hours after operation or death was washed, minced, boiled with distilled water, extracted overnight in the cold, and then centrifuged. The supernatant fluid was used.

The diagnosis of all specimens was based on microscopic and/or gross anatomical findings.

Purification of Mucoid Material.—(a) Meconium. Fractionation of a meconium pool was carried out following approximately the procedure described by Buchanan and Rapoport.[3] Five hundred milliliters of a suspension of pooled meconium, 3.7 per cent d.w., blood group A (H), was buffered with acetic acid and sodium acetate, 0.2 M, to pH 4.85. Fractionation with ethanol was conducted at +4° C. and the precipitate centrifuged at 4,000 r.p.m. for fifteen minutes at 0° C. (b) Pseudomucinous ovarian cyst fluid. A simple alcohol fractionation as for meconium did not yield satisfactory results due to the high protein content of the cyst material. Better separation was obtained by following in a general way a method described by Aminoff, Morgan, and Watkins[4] for the isolation of group A substance. Each cyst, however, necessitated appreciable variations in the purification procedure because of differences in the proportions of active and inert material as well as in the consistency of the individual cyst fluids. (c) Mucoid material from cattle. The method used for meconium proved to be the most convenient of several tried for the purification of mucoid material from the fourth stomach and small intestine of cattle. These materials were obtained from freshly slaughtered animals. After washing the organs and grinding them in a Waring blendor, an aqueous extract was prepared and heated to boiling. The sediment was removed by centrifugation. The supernate was brought to 1 per cent concentration before fractionation. (d) Frog spawn mucin. This material was obtained from *Rana clamitans.* The starting material was freed of organic debris and water as completely as possible. The mucin was separated from the embryos following the procedure of Folkes, Grant, and Jones.[5] The mucin was finally obtained in the form of black flakes, which were water insoluble. For the microbiologic and blood group tests this material was dissolved in 0.05 N NaOH and then neutralized.

All mucoids were dialyzed before use.

TABLE II. DISTRIBUTION OF BIFIDUS FACTOR AND BLOOD GROUP MUCOIDS IN WATERY EXTRACTS OF HUMAN TISSUES, MALIGNANCIES, AND SOME OTHER PREPARATIONS

SOURCE	PREPARATION	BIFIDUS FACTOR (MG./UNIT)	BLOOD GROUP ACTIVITY*		
			A_1	B	H†
Human	Submaxillary and sublingual gland	4		+++	
	Stomach‡	7.5	+++		
	Small intestine	4			+++
	Kidney§	7			+
	Lung	20	+	+	+
	Spleen‡	25	−	−	+
	Pancreas	19	+	++	+
	Transverse colon‖	15	+		+
	Prostate‡	25	+		
	Carcinoma of pancreatic duct: with metastasis into liver	2	++	−	++
	Carcinoma of breast (3 types)	av. 12	av. +	−	−
	Carcinoma of colon	12	+	−	−
	Carcinoma of rectum	inactive			
	Carcinoma of lung	inactive	−	(+)	−
	Sero-mucoprotein	inactive	+	−	−
	"Intrinsic Factor"	0.6	++++	++(++)	++
Animal	Hog gastric mucin[1]	0.6	+++	−	+++
	Blood group substance[2] A	0.7	++++	−	+++
	Blood group substance[2] B	0.7	++	++++	+++
	Cattle small intestine¶	3.3	++++	+	++
	Cattle abomasum pyloric region	2.5	++	++	+++
	Cattle abomasum fundus region	4.6-6.1	++	++	−
	Ethanol (50-60%) fraction of whole cattle abomasum exract	0.5	+++(+)	++	+++
	"Rennet"[3]	23	+++	++	++
	"Rennin"[4]	inactive	++(+)	++	++
	Forssman Hapten	inactive	++	−	−
	Frog spawn mucin	6.5	+++	++	+++
	Oyster, extract of whole	40	++	·	+

*Recipr. inhibition titers: + = 10^2–10^3; ++ = 10^3–10^4; +++ = 10^4–10^5; ++++ = 10^5–10^6.

†Eel antiserum.

‡Only one organ tested instead of two to three as usual.

§After dialysis.

‖Ascending colon had the highest, sigmoid the lowest activity; extracts of the descending colon were sometimes toxic.

¶Dialysis increased the total bifidus activity of the remaining material three to fourfold.

[1]Wilson Laboratories, Chicago, Ill.

[2]Sharp & Dohme, Philadelphia, Pa.

[3]G. B. I., Chagrin Falls, Ohio. "Rennet Armour" had similar properties.

[4]Delta Chemical, New York, N. Y.

TEST METHODS

Assay for Bifidus Factor.—The microbiologic activity was determined by means of potentiometric titration of the acid produced by the organism in 10 ml. of medium after forty hours' incubation.[6] A substrate was called inactive when the unit was greater than 40 to 50 mg. dry weight. One unit is defined as that amount of test substrate which gives half maximal growth within forty hours. It corresponds to 0.06 ml. skimmed human milk added to 10 ml. fluid medium.[6]

Determination of Serologic Activity.—The agglutination inhibition test[7] was employed. Progressive dilution of the agglutinogen and a constant concentration of antiserum were used. Freshly drawn erythrocytes washed with 0.5 per cent NaCl and centrifuged three times were used in the test for A, B, and H agglutinogens. In testing for the presence of Le^a substance, the cells were washed only once as the Le^a characteristic is partly washed off.[8] One-tenth milliliter of saline, antigen solution, and antiserum were used for all tests. The same volume of a 0.5 per cent solution of erythrocytes was used for the "A and B test." For the "Le^a and H tests," the erythrocytes were added in 0.02 ml. saline in 2.5 per cent

concentration to avoid unnecessary dilution. Twofold geometrical dilution was used throughout. The titrations were done at room temperature (22° to 25° C.), with the exception of the Lewis test, which was done at 14° to 16° C. The end points were read two hours after the addition of erythrocytes. In the case of the Lewis series, it was necessary to centrifuge (1,000 r.p.m. for one minute) before reading, because of weak agglutinations. A blank and a negative control were always run simultaneously. At intervals, a positive control was included. Since the range between beginning and complete agglutination showed great variation (over 100 per cent), reading of macroscopic agglutination proved to be inaccurate. The lowest dilution in which there was agglutination of at least 2 to 3 cells in several places in one low-power field of the microscope was taken as the agglutination end point.

All titers are based on 100 per cent dry matter and represent an average of at least two determinations. Titers lower than 1:20 to 1:40 are not considered in this study. In order to obtain good sensitivity and sufficient range of titers in the hemagglutination inhibition assays, all test sera were brought to a concentration corresponding to approximately 8 to 16 minimum hemagglutinating units[9] under the conditions employed.

Test Sera.—Sterile anti-A and anti-B immune sera of human origin were used* (titer for both sera, 1:256). The anti-Le^a serum from a woman, blood group A_1, Rh negative (no anti-Rh titer), was not diluted before use.† The anti-H (O) sera were of animal origin (they were used against O cells, exclusively). Heterologic anti-H serum from ten common eels (*Anguilla anguilla*) was obtained according to the procedure described by Jonsson.[10] The individual sera, which all had approximately the same titer, were pooled, thoroughly adsorbed with A_1 B cells, and heated for thirty-five minutes at 56° C. The titer against O cells was then 1:1,024. Immune anti-H serum prepared from the rabbit[11]‡ had an original titer of about 1:8,000.

All sera were stored at –15° C. in small portions, each sufficient for one or two assays. No preservative was added. The activity was tested before each assay.

Physical and Chemical Determinations.—Nitrogen was determined on lyophilized material with the micro-Kjeldahl method according to Pregl and Roth. The relative viscosity of a 0.5 per cent solution of test material in 0.85 per cent saline was determined at 37° C. in an Ostwald viscosimeter.

RESULTS

Human Secretions.—All epithelial mucinous secretions of man which were examined (Table I) contained water-soluble blood group activity and Bifidus Factor. The concentration of Bifidus Factor is of the same order of magnitude in all active secretions, with the exception of amniotic fluid. The blood group activities, however, show wide variations, at least as far as individual characteristics are concerned. They are low in colostrum and milk. Neither Bifidus Factor nor blood group activity has been found in any significant concentration in spinal fluid nor are they contained in pleural, pericardial, peritoneal, or synovial fluid, which are considered to be mesothelial secretions. Contents of serous and follicular ovarian cysts were inactive as was the fluid from a mediastinal teratoma. Secretions from two malignant mucinous ovarian cysts contained no Bifidus Factor. The contents of these cysts resembled those first described by Mitjukoff,[12] as "trembling jelly" type. Human seromucoprotein§ was an exception, as it had an A titer of 1:400, but revealed no growth-promoting properties for *L. bifidus* var. *Penn.*

*Thanks for these sera are due to Dr. N. Abelson, Children's Hospital, Philadelphia, Pa.
†Kindly given repeatedly by Dr. A. Richardson Jones, Blood Grouping Laboratory, Boston, Mass.
‡Most kindly given repeatedly by Dr. W. T. J. Morgan, Lister Institute, London, England.
§Kindly given by Dr. R. Winzler, Department of Biochemistry, University of Illinois.

Of twenty salivas tested, all were highly bifidus-active, but five were without A B H characteristics. These five salivas from nonsecretors, however, possessed the Le^a property. A number of other secretions were also found without A, B, or H activity. The secretors as well as the nonsecretors included individuals of A, B, and O blood groups.

Thirty meconia were tested for A B H activity and eleven of these also for Le^a characteristics. The distribution of blood group A and B was in good agreement with the data published by Buchanan and Rapoport.[13] In no instance was a meconium encountered not possessing at least one of the four blood group characters. The H activity, especially when in combination with A or B, showed extreme variation in its concentration. In about half of the cases tested, it inhibited only to a dilution of 1:100; although, in a few instances when H occurred alone, the inhibition end point was found at a dilution of 1:1,600,000. All meconium samples tested were found to contain Bifidus Factor in high, though variable, concentration. Meconium obtained from a meconium ileus showed no difference from normal samples.

In contradistinction to colostrum and milk, where a large proportion of the Bifidus Factor is diffusible, Bifidus Factor in mucoid secretions as described previously appears to be nondiffusible. Dialysates of saliva and pseudomucinous ovarian cyst fluid contained less than 2 per cent of the total bifidus and blood group activity of these materials.

Moderate concentration of Bifidus Factor and an appreciable serologic titer for A and B were found in a pool of human sweat.

Extracts of Organs.—As with the secretions of glandular organs, water-soluble blood groups and bifidus activity occurred together and were obtained in significant amounts only from those epithelial organs which secrete mucus (Table II). The most active tissues, therefore, were found to be the submaxillary-sublingual gland, the stomach, and the duodenum. Thyroid, adrenal, testis, ovary, liver, brain, and skeletal muscle did not contain blood group or bifidus activity in any appreciable concentration. There was no qualitative difference between malignant tumors and the normal structures of the same organs from which the tumors arose. Of eight preparations of purified intrinsic antipernicious anemia factor,* all showed bifidus and A and H blood group activity comparable to that of gastric mucin. B activity was variable.

Material From Animal Sources.—Mucoid-containing substrates of animal origin in all but two instances contained Bifidus Factor and blood group substances together. Forssman hapten (obtained from sheep erythrocytes†) up to a dilution of 1:1,600 inhibited agglutination of A_1 cells but did not promote growth of *L. bifidus* var. *Penn.* The other exception was one of three rennin preparations tested (Table II).

Blood group A and B preparations of pig and horse origin, respectively, had high bifidus activity. Both preparations showed marked H activity and the

*One preparation was generously donated by Dr. G. B. Glass, New York Medical College, N. Y. Seven preparations, of which three were electrophoretically homogeneous, were kindly furnished by Dr. T. Jukes, Lederle Laboratories, N. Y.

†Generously donated by Dr. M. M. Mayer, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

B substance also contained some A characteristic. Assays of aqueous extracts of stomach and small intestine of cattle showed that the highest bifidus and H activity was located in the pyloric region, whereas the small intestine had lowest bifidus and highest A activity (Table II). The concentration of blood group A, B, and H substances and of Bifidus Factor was constant throughout the small intestine, which has a total length of about 30 meters. The frog spawn mucin preparation had moderate microbiologic activity and blood group titer for A and H as well as a low B titer. Dialyzed water extracts of whole oysters showed that *Mollusca* also produce Bifidus Factor and blood group substances (Table II).

Purified Mucoid Preparations.—In fractionation of meconium after removal of inactive bulk, the most active and largest fraction was obtained at ethanol concentrations of 50 to 67 per cent (Table III). A and H properties as well as Bifidus Factor have their greatest activity in this range. At higher ethanol concentrations, there is a sharp decrease of blood group activity, while bifidus activity declines much more slowly. The fraction of cattle mucoid material precipitating in 50 to 60 per cent ethanol contained the Bifidus Factor in its highest activity and largest amount (Table II). The H and B activities, which in general were only moderate, also had their highest titer in this fraction, whereas more than 50 per cent of A-active material precipitated at a concentration of ethanol below 50 per cent.

TABLE III. DISTRIBUTION OF BLOOD GROUP AND MICROBIOLOGIC ACTIVITY IN ETHANOL FRACTIONS OF MECONIUM A (H) POOL

FRACTION	ETHANOL PER CENT	DRY WEIGHT OF PRECIPITATE (GM.)	RECIPROCAL OF INHIBITION TITER × 10^2		BIFIDUS FACTOR (MG./UNIT)
			A	H†	
Starting material	-	15	1,024	8	1.2
Dialyzed starting material*	-	-	4,096	16	0.6
I	46	ca.11	256	2	4.2
II	50	0.58	4,096	16	0.6
III	60	1.63	32,000	32	0.4
IV	67	1.00	32,000	32	0.4
V	75	0.34	1,024	1	0.7
VI	85	0.28	16	<1	0.95

*Dialyzed against 20 vol. of distilled water for 48 hours at +2° C.; proportion (on weight basis) of dialyzable to nondialyzable material = 1/20.

†Eel antiserum.

It was observed that mere dialysis of crude material such as meconium or cattle mucoid often increased the total bifidus and blood group activity by 100 to 400 per cent.

The characteristics of the fractions obtained on purification of pseudomucinous ovarian cyst fluids are outlined in Table IV. One example is presented for each main blood group. The A and B cysts contained some H activity, and the H cyst low A activity. These additional characteristics amounted to about 0.01 to 0.1 per cent of that of the main blood group property. On purification of the cyst fluids, the fractions with the lowest nitrogen content 3.6 to 4.6 per cent had the greatest bifidus and blood group activity. On the basis of weight, the purest H and B materials are seven to ten times more active as Bifidus Factor than the A material.

TABLE IV. DISTRIBUTION OF BIFIDUS FACTOR AND BLOOD GROUP MUCOIDS IN FRACTIONS OF PSEUDOMUCINOUS OVARIAN CYST FLUID

BLOOD GROUP OF CYST FLUID	FRACTION	DRY WEIGHT* (GM.)	BIFIDUS FACTOR (MG./UNIT)	RECIPROCAL OF INHIB. TITER × 10^2			N PER CENT	RELATIVE VISCOSITY
				A_1	B	H†		
O (H)	0	325	1.1	<1	<1	2,048		
	1	314	3.4					
	2	10.5	0.3	1	<1	4,096		
	3	0.52	0.95	4	<1	64	8.00	1.05‡
	4	3.5	0.15	16	<1	8,192	3.62	1.25‡
B	0	85	1	<1	64,000	64		
	1	80	1.3	<1	8,192	16		
	2	3.3	0.2	<1	128,000	128	5.72	1.30
	3	0.22	1.6	<1	128	64	9.25	
	4	1.92	0.13	<1	256,000	128	4.08	1.27
A	0	140	2.5	1,600,000	<1	1,024		1.76
	1	133						
	2	6.45	1.3	6,400,000	<1	1,024	6.48	
	3	0.35	1.3	3,200,000	<1	1,024	6.60	1.24
	4	4.59	1.2	12,800,000-25,600,000	<1	2,048	4.60	1.29

*All fractions were dialyzed before determination, except the starting material. Fraction I was estimated.

†Eel antiserum.

‡After 1-week storage of the solution at −10° C.

Description of Fractions.—Fraction numbers: 0. Starting material. 1. Precipitate by 10 per cent trichloroacetic acid. 2. Ethanol precipitate of the supernatant fluid. 3. Phenol extract of 2. 4. Water extract of phenol insoluble material of 2.

Comparison of the serologic activities gives an almost reverse picture. Here the A material was very active. Complete inhibition toward A_1 cells was found at a concentration of less than 0.01 microgram per milliliter. The inhibitory power toward A_2 cells was even higher, as could be expected, and increased with increasing purity of the material. For the purest material from B and H cysts, the smallest amount of mucoid giving complete inhibition under standard conditions was of the order of 0.1 and 1 microgram per milliliter, respectively. The purest preparations of all 3 cysts were found to contain less than 0.01 per cent sulfur. The phosphorus content was 0.11, 0.18 and 0.41 per cent for the B, H, and A substances.

Purified substances were uniformly less viscous than the starting material and the viscosity of the most active substances was about the same for each cyst.

One per cent aqueous solutions of these purified mucoids formed no precipitate on boiling and gave a negative reaction in the ring test with 10 per cent sulfosalicylic acid. The ninhydrin, biuret, xanthoproteic, Millon-Cole, Hopkins-Cole, and Seliwanoff tests were negative. The Molisch test was strongly positive. With the modified Bial test[14] a violet color was obtained. The color was taken up entirely in the n-amyl alcohol layer when the sample was shaken with this compound. The Ehrlich reaction (p-dimethyl-amino-benzaldehyde) was faintly positive upon heating. Previous boiling of the test material with 2 N sodium carbonate rendered this test strongly positive.

The most active fractions from meconium did not have as low a protein content as the material from ovarian cysts since the ninhydrin, xanthoproteic, Millon-Cole, and Hopkins-Cole tests were positive, and the sulfosalicylic acid and biuret tests faintly so.

Paper chromatographic analysis of acid hydrolysates of the purest preparations from cysts and meconium was carried out as described in the subsequent paper.[14a] Fucose, hexosamine, and and galactose were found in all instances.

DISCUSSION

Bifidus activity as it occurs in man may be associated with larger or smaller molecules, of which the former seems to be closely related to blood group substances. In colostrum and milk, over 50 per cent of the bifidus activity is diffusible[15] and blood group activity is low. In epithelial mucoid secretions, on the other hand, the Bifidus Factor is diffusible to less than 2 per cent and is associated with moderate to high blood group titers. In general, on purification of mucoid materials those fractions which showed the highest blood group activity were also most active as Bifidus Factor. In several instances, however, (e.g., Table III) fractions precipitated at high alcohol concentration showed relatively greater bifidus than blood group activity. Such fractions possibly contain partially degraded blood group substance. That they should be inactive as blood group substance and active as Bifidus Factor is in accord with the observation that the former property is usually limited to compounds of very high molecular weight,[4, 16] whereas bifidus activity is shown by large or small molecules containing N-acetyl-D-hexosamine in appropriate linkage from the blood group substances themselves through the diffusible oligosaccharides of milk down to β-methyl-N-acetyl-glucosaminide.[17]

It has not been possible to relate the Bifidus Factor to one specific blood group polysaccharide. The results suggest, however, that bifidus activity in man may be more closely associated with B and H activity than with the A group. The bifidus activity of the purified preparation from a cyst with blood group A activity was about 10 times lower on a nitrogen and weight basis than that of corresponding fractions from cysts of blood groups B and H activity. On the other hand, the agglutination-inhibiting power of the A mucoid was very much higher than that of the purified B material tested under analogous conditions. Similar results for the Bifidus Factor were obtained with highly purified, essentially homogeneous A, B, and Le^a preparations made available to us through the kindness of Professor W. T. J. Morgan.* The nitrogen content of all three preparations was the same, and the hexosamine content of the A substance was somewhat higher than that of the others. The lower activity of the A mucoid may find its explanation in a different structural arrangement of this mucopolysaccharide. Morgan and his group found the A substance to be less labile than the Le^a and H substances.[16] Enzymatic studies which have been reported[14a] indicate that the H substance may be especially accessible for *L. bifidus* var. *Penn.*

High bifidus and blood group activity appear to be associated with structures containing globlet cells and with the mucinous secretions elaborated by

*Preparations from ovarian cyst fluid, kindly furnished by Professor W. T. J. Morgan, Lister Institute, London, England, showed one unit of Bifidus Factor in 0.25 mg. of Le^a substance, 0.25 mg. of B substance, and 1 mg. of A substance. The analytical data for these materials as given by Prof. Morgan are:

Le^a: N, 5.3%, Fucose 13.0%, Hexosamine$^+$) 32%, Reducing sugars^{++}) 57%
B: N, 5.3%, Fucose 16.9%, Hexosamine$^+$) 22%, Reducing sugars^{++}) 50%
A: N, 5.3%, Fucose 18.0%, Hexosamine$^+$) 35%
$^+$) as base, $^{++}$) in terms of glucose.

these structures. With the exception of amniotic fluid, the Bifidus Factor is present in approximately the same concentrations in all active human secretions. Blood group activity shows wide variations. Several different blood group mucoids, however, are often present in the same secretion and there may be still some unknown blood group mucopolysaccharides. This has been suggested by Morgan.[18] It seems to be permissible to assume the presence of the Le^a property in those instances where individual epithelial secretions were found free of A, B, and H activity. In all such instances, Le^a factor was found when tested for. No mesothelial secretion contained either Bifidus Factor or blood group activity in any significant amount.

Goblet cells and their secretions tested so far give a positive response to the Hotchkiss-McManus histologic test[19, 20] for free α-glycols indicating with a high degree of probability the presence of polysaccharides.

All organs tested which contained goblet cells were active. Only the activity of colon was low and decreased toward the rectum. This may be explained by the action of blood group destroying enzymes described by Schiff and Akune[21] and Witebski and Neter.[22]

Prostate and spleen, which do not contain goblet cells, had slight bifidus and blood group activity, which may be explained in the first case by the presence of some duct and gland structures, mainly seminal vesicles which cannot be completely separated, and in the second by some more water-soluble blood group substance, like the Le^a of disintegrated erythrocytes. No explanation can be offered for the fairly high activity of kidney extracts.

With respect to the question of the origin of blood group activity[13] and bifidus activity of amniotic fluid, one may assume that the bifidus and blood group characteristics in amniotic fluid have their origin in the meconium, as the blood group and bifidus activity is considerably greater in meconium than in amniotic fluid. It is not known with certainty which cells elaborate bifidus and blood group factors in the mammary gland. Two points should be considered: first, mucoid carcinomas of the mammary gland are well known and thought to arise from the ducts; second, apocrine gland cells have much in common with goblet cells. We found that sweat, the secretion of apocrine glands, contains moderate bifidus and blood group activity. Mammary gland cells are related to apocrine glands. The occurrence of Bifidus Factor and blood group A, B, and H characteristics in the mucoid secretions of the goblet cell-containing oviduct of a lower vertebrate such as the frog, *Rana clamitans*, demonstrates the wide distribution of these characteristics. That they are not limited to the vertebrates is proved by their occurrence in *Mollusca* such as *Ostrea*.

Human seromucoprotein and Forssman hapten from sheep erythrocytes both show A activity of low and moderate degree, respectively. No other blood group nor bifidus activity for these substances could be detected. The Forssman hapten, which is originally not water soluble, is chemically and serologically related to, but not identical with, the A blood group mucoid.[23] The seromucoprotein is chemically quite different from the blood group mucoids.[24] It may contain merely traces of the blood group A mucopolysaccharide, which are

detectable only with the very sensitive agglutination inhibition test. It was of interest that three rennin preparations tested contained blood group activity but only two of them Bifidus Factor. In the case of the inactive preparation, it is possible to assume the influence of some inhibitor. This view is favored by the finding that crude meconium and cattle mucoid often showed an increase of their over-all blood group and bifidus activity, if dialyzed or purified.

Appreciable bifidus activity and blood group titer were found in highly purified, some of them electrophoretically homogeneous, "intrinsic factor" preparations. This suggests an association between these properties which may extend to compounds of similar activity as found in colostrum or milk.

SUMMARY

The distribution of water-soluble blood group mucoids in human and some animal secretions and tissues was investigated, and their function as growth factor for *Lactobacillus bifidus* var. *Penn* studied. These substances are secreted by goblet cell-containing structures. They have been found to be widely distributed in the animal kingdom from human beings to *Mollusca*.

A considerable purification of some mucoid secretions was achieved by fractionation procedures. High blood group activity was always accompanied by a high concentration of Bifidus Factor. Considerable microbiologic activity, however, persisted in fractions precipitated by high concentrations of ethanol, while blood group activity decreased rapidly. Differences in the bifidus activity of the different blood group substances were noted. Highly purified "intrinsic factor" preparations uniformly were associated with high bifidus and blood group activity.

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ENZYMATIC INACTIVATION OF BIFIDUS FACTOR AND BLOOD GROUP SUBSTANCES

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NONDIALYZABLE compounds, such as purified blood group substances or the active materials present in hog mucin, have been found to exert high growth-promoting activity for *Lactobacillus bifidus* var. *Penn*.[1] The assumption was made that these large molecules are first broken up into smaller, microbiologically still active components by enzymes of *L. bifidus* var. *Penn* before they may pass through the cell wall. This hypothesis was tested in vitro by observing the action of cell extracts prepared from *L. bifidus* var. *Penn* and from other strains of *L. bifidus* on various sources of the Bifidus Growth Factor. This study included not only microbiologic assays for Bifidus Factor, with and without preceding dialysis, but also serologic titration of blood group activity, and in several instances determination of free reducing sugar and chromatographic analysis.

Enzymatic inactivation of blood group substances, measured by their specific serologic properties, was first observed by Schiff and his associates[2-6] as well as by Landsteiner and Chase,[7] and later intensively studied, using highly purified blood group preparations, by Morgan and his collaborators.[8, 9] Such enzymes were found in cultures and culture filtrates from certain strains of *Clostridium welchii* as well as in human saliva and feces. Inasmuch as, in general, substances of high blood group activity are also active as Bifidus Factor,[1] it appeared to be of interest to investigate whether enzymes known to inactivate blood group substances serologically may also destroy the microbiologic activity.

Control studies with several other enzymes were also made.

MATERIALS AND METHODS

Enzyme preparations were obtained from *L. bifidus* var. *Penn*, grown for forty-eight hours in the usual semisynthetic medium[10] with the addition of 2 per cent of skimmed human milk. The centrifuged cells were extracted with 0.01 M phosphate buffer, pH 6.5 following the procedure of Cohen.[11] In several instances, the extract was used directly; in most cases, it was preserved by drying from the frozen state and later dissolved for assay in distilled water. Enzymatic activity was found in the cell extract, but not in the culture filtrate. No purification or fractionation of this crude cell extract was attempted. About 1 mg. of the dried preparation equivalent to about 0.25 mg. of protein was used for each unit of microbiologic activity of the substrate.

Extracts were also prepared in the same manner from a strain (''Jackson'') of regular *L. bifidus*.[10]

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This paper is dedicated to Professor Otto Hahn, President of the Max Planck Society. It will be incorporated in a special volume on the occasion of Professor Hahn's seventy-fifth birthday.

*Scientific Member of the Max Planck Society.
**Woodward Fellow in Physiological Chemistry.

Of the three Clostridium strains tested, *Cl. perfringens* Type B, A.T.C.C. No. 7907 furnished the most active culture filtrate, from which potent enzyme preparations were made by precipitation with acetone.[9] In contrast to *L. bifidus* var. *Penn*, in Clostridium the activity was represented by ecto-, and not by endo-enzymes.

Saliva was obtained from healthy individuals at least two hours after the last meal. Secretion was stimulated by chewing gum. The specimens were stored in the refrigerator for not more than two days.

A number of other enzymes were tested including preparations from *Aspergillus oryzae*,* *Aspergillus flavus*,* *Rhizopus delamar*,* *Bacillus subtilis** and *Vibrio cholerae*,† as well as lactase, pectinase,* hyaluronidase, emulsin, mucinase,‡ influenza virus,† β-amylase, and trypsin. None was able to inactivate the Bifidus Factor or the serologic A, B, and H characteristics of the blood group mucoids. In addition, no indication was found that any of these enzymes would split (depolymerize) large molecules into active breakdown products.

For measurement of enzymatic activity, the material to be tested was incubated with active and, as control, with boiled enzyme preparation at 37° C., under toluene, and aliquots were removed at the indicated times. No standard enzyme concentration was used, but care was taken that the enzyme concentration was never the limiting factor in the experiment. The substrate concentration was usually kept within a range of 2 to 4 growth units[12] per milliliter of the enzyme-substrate mixture. The enzymatic process was terminated by heating the mixture for ten to fifteen minutes in boiling water. For the microbiologic assay, toluene was removed by drying the sample on a steam bath and reconstituting to the appropriate volume with distilled water. When necessary, pH was adjusted to 6.5.

The following methods were used for determination of the extent of enzymatic activity:

1. Microbiologic assay for Bifidus Factor.[12]

2. The agglutination inhibition test.[1] The twofold geometrical dilution procedure was used throughout. Lowering of the inhibition end point by 4 tubes corresponds to 94 per cent inactivation of serologic activity.

3. Determination of carbohydrate breakdown products. Free reducing sugar (expressed as glucose) was measured by the method of Folin and Malmros.[13] The nature of the sugars liberated was investigated by chromatographic analysis, with simultaneous use of reference standards. For chromatography the substrates were dialyzed prior to incubation with enzyme. The one-dimensional descending method and Whatman paper No. 1 were employed with the following solvents[14]: n-butanol [4], glacial acetic acid [1], water [5]; n-butanol [6], pyridine [4], water [3]; and ethyl acetate [2], pyridine [1], water [2]. The dried paper strips were sprayed with the following reagents: (a) anilinoxalate for detection of sugars; (b) Morgan-Elson reagent[14] for hexosamines and N-acetyl-hexosamines; and (c) ninhydrin[14] for amino acids and amino sugars. The samples to be tested in chromatographic analysis were shaken with 0.08 Gm. powdered Amberlite MB2 per milliliter for ten minutes to remove inorganic salts, centrifuged, and concentrated to the required volume. As long as stronger electrolytes were present, glucosamine was not removed by the resin used.

TESTS WITH BIFIDUS ENZYME

Meconium.—Meconium of blood group B, collected fresh from 1- to 2-day-old normal infants, was pooled, diluted with water to 1 per cent dry weight, dialyzed, and autoclaved. Aliquots were diluted with an equal volume of 0.01 M phosphate buffer of varying pH. Samples at each pH were incubated forty-eight hours with active and boiled enzyme, with simultaneous dialysis against 10 volumes of the same phosphate buffer. The results summarized in Table I permit the following conclusions: bifidus enzyme inactivates the Bifidus Factor

*Kindly furnished by Dr. E. Snyder, Takamine Laboratories, Clifton, N. J.
†Kindly furnished by Dr. J. Seifter, Wyeth Institute, Philadelphia, Pa.
‡Kindly given by Dr. W. Henle, Children's Hospital, Philadelphia, Pa.

TABLE I. INFLUENCE OF pH ON THE INACTIVATION OF MECONIUM (BLOOD GROUP B) BY BIFIDUS ENZYME PREPARATION

INCUBATION TIME: 48 HOURS

pH	BIFIDUS FACTOR ACTIVITY ML./UNIT IN				REDUCING SUGAR MG./ML.		SEROLOGIC TITER (RECIPR.)‡	
	DIALYZED RESIDUE WITH		DIALYSATE* WITH		DIALYSATE*		DIALYZED RESIDUE	
	ACTIVE ENZYME	BOILED ENZYME	ACTIVE ENZYME	BOILED ENZYME	ACTIVE ENZYME	BOILED ENZYME	ACTIVE ENZYME	BOILED ENZYME
2.5	0.38	0.38	2.0	2.5	0.09	0.10	–	–
4.0	0.38	0.23	3.5	2.5	0.11	0.11	64	–
5.5	1.40	0.45	6.0	2.0	0.23	0.15	8	64
7.0	1.40	0.38	4.0	2.5	0.16	0.07	–	–
8.5†	0.70	0.55	5.0	4.0	0.15	0.06	32 (16±)	128 (64±)
10.0†	0.50	0.55	3.0	2.5	0.10	0.09	64	64

*Dialysate has been concentrated to original volume.

†During incubation pH changed from 8.5 to 8.1 and from 10.0 to 9.3, respectively.

‡"Serologic titer (recipr.)" expresses in this and the following tables the reciprocal of the dilution of agglutinogen, which will give complete inhibition of agglutination under the conditions of the test.

and the blood group characteristics, the most effective pH range being from 5.5 to 7.0. The Bifidus Factor, as it occurs in meconium, is nondialyzable and there is no indication that depolymerization into diffusible active components takes place under the action of the enzyme preparation. In a similar experiment, reducing sugar was determined in the dialysate. Inactivation was accompanied by liberation of free reducing sugar.

TABLE II. INACTIVATION OF BIFIDUS FACTOR AND OF BLOOD GROUP SUBSTANCES IN MECONIUM BY BIFIDUS ENZYME PREPARATION

INCUBATION TIME (HOURS)	SEROLOGIC TITER (RECIPR.)			INACTIVATION OF BIFIDUS FACTOR (%)			REDUCING SUGAR* (MG./ML.)	
	A	B	O (H)	A	B	O	A†	B†
0	1,600	512	1,024	–	–	–	0.82	0.45
4	1,280	256	64	72	44	22	1.16	0.75
8	800	32	8	72	44	–	1.42	0.84
24	800	32	4	86	75	50	–	0.90
48	400	16	4	84	87	73	1.46	1.10

*Expressed as glucose.

†The average increase in reducing sugar per unit of Bifidus Factor inactivated is 196γ for blood group A substance and 135γ for blood group B substance.

In other experiments, samples of meconium of different blood groups were treated with enzyme for varying periods of time (Table II). Microbiologic activity and serologic blood group titer decreased progressively with simultaneous liberation of sugar. In general, bifidus enzyme rapidly inactivated the H (O) property, in accordance with the known lability of the H substance.[16] Microbiologic growth activity decreased more slowly. The amount of reducing sugar liberated was calculated to be between 125 and 200γ per unit of Bifidus Factor inactivated. Similar values were found in other experiments.

Purified Blood Group Substances.—Material of high activity with respect to Bifidus Factor (1 unit in 200γ and with a serologic titer of 1:6,400,000 for blood group substance B was prepared from a pseudomucinous ovarian cyst.*

*Our preparations compare favorably with those obtained from Professor Morgan, London, England. (cf. Table IV.[1])

On treatment with bifidus enzyme, this substance was inactivated as was the crude meconium. The serologic titer for B substance was reduced from the initial value of 1:4,096 for the dilution used to 1:2,048 after sixteen hours and to 1:512 after forty-eight hours. After the same periods of time, the microbiologic activity had been reduced by 25 and 72 per cent, respectively.

Similar inactivation was observed with blood group substances of animal origin (Table III). In analogy to the observations on meconium, blood group substance H is preferentially destroyed by the bifidus enzyme, together with the microbiologic activity, whereas blood group substances A and B are characterized by greater resistance to the enzyme. The agglutinogens A and B of animal origin were often degraded only to an insignificant extent during the period of incubation.

TABLE III. INACTIVATION OF BLOOD GROUP SUBSTANCES AND OF BIFIDUS FACTOR OF ANIMAL ORIGIN BY BIFIDUS ENZYME PREPARATION

SUBSTRATE	INCUBATION TIME (HOURS)	SEROLOGIC TITER (RECIPR.) A	B	H	BIFIDUS FACTOR ML./UNIT	DECREASE OF ACTIVITY (PER CENT)
Purified B	0	–	512	128	0.37	–
(Sharp & Dohme)	4	–	512	4	0.43	14
0.25%	16	–	128±	1	0.5	26
	48	–	16	1	1.2	69
Hog mucin*	0	256	–	512	0.75	–
(Wilson)	4	256	–	4	1.3	42
0,25%	16	–	–	2	1.5	50
	24	128	-	1	2.8	73
	48	128	-	1	3.5	78
Purified A*	0	8,200	-	–	0.47	–
(Sharp & Dohme)	4	4,096	–	–	0.65	28
0.25%	16	2,048	–	–	1.10	57
	48	2,048	–	–	inactive	>80

*The decrease in A titer is probably not significant.

Human Milk.—The bifidus enzyme also inactivates the Bifidus Factor in human milk and in active fractions prepared from deproteinized human milk by adsorption on charcoal and consecutive elution with 20 per cent acetic acid.[15] In one experiment, the activity of human milk was reduced by 90 per cent, from 0.1 ml. per unit to 1.0 ml. per unit, when treated with bifidus enzyme for forty-eight hours. The activity of control samples treated with boiled or autoclaved enzyme was unchanged during the same period of time. Corresponding results with a milk eluate are summarized in Table IV. As with meconium, inactivation of the Bifidus Factor is accompanied by the liberation of reducing sugar: approximately 175 γ of the sugar are released per unit of activity lost.

Chromatographic Analysis of Enzymatic Breakdown Products.—Tests carried out on dialysates of enzymatically inactivated purified blood group substances and meconium revealed the presence of fucose, galactose, and N-acetylhexosamine. These monosaccharides were not present in the enzyme preparation itself nor in samples of the substrate incubated with boiled enzyme. With meconium, where the enzyme reaction was allowed to take place over a wide

TABLE IV. INACTIVATION OF ELUATE FROM HUMAN MILK BY BIFIDUS ENZYME PREPARATION

INCUBATION TIME (HOURS)	BIFIDUS FACTOR		REDUCING SUGAR* (MG./ML.)
	ML./UNIT	DECREASE OF ACTIVITY (PER CENT)	
0	0.16	—	0.55
2	0.21	24	0.82
4	0.30	47	0.87
8	0.27	40	1.05
24	0.45	65	1.20
48	0.47	66	1.50
64	0.85	81	1.70

*Expressed as glucose.

pH range, the highest concentration of sugars was observed between pH 5.5 and 6.5. At pH 7, no N-acetyl-hexosamine was detected and the amount of other sugars was visibly reduced. Free glucosamine was never detected. At pH 5.5-6.5, an aniline oxalate-positive spot with an Rf less than that of lactose was often observed. It probably represents an oligosaccharide. When milk eluate was used, glucose also was detected. Since the milk eluate was not entirely free of lactose, which is also hydrolyzed by the enzyme preparation, some of the glucose and galactose must have originated from this sugar. Acid hydrolysis of highly purified samples has indicated that milk eluate contains both glucose and galactose.[15]

Enzymes of a "Regular" Strain of L. bifidus.—Enzyme preparations obtained from a regular strain ("Jackson") of *L. bifidus,* not requiring the specific growth factor present in human milk, produced no inactivation of serologic blood group activity or of the Bifidus Factor in the various sources tested: human milk, milk eluate, meconium, purified blood group substances.

TESTS WITH CLOSTRIDIUM ENZYME

Ecto-enzyme preparations from *Cl. welchii* Type B were tested for inactivating capacity on various compounds exhibiting serologic blood group and Bifidus Factor activity. Representative examples are contained in Table V. The speed of inactivation of the Bifidus Factor in purified blood group substances is comparable to that obtained with "Bifidus enzyme." In contrast, the destruction of serologic blood group properties for A and B proceeds faster and reaches usually 90 per cent reduction of the original titer within the first four hours.

In crude extracts of meconium, boiled or dialyzed, our Clostridium enzyme preparations were ineffective both with regard to inactivation of the Bifidus Factor and of the serologic blood group titer. Apparently, such extracts contain an inhibitor. A purified meconium fraction, obtained through alcohol precipitation, lost the original inhibitor and responded to treatment with Clostridium enzyme by almost complete inactivation of Bifidus Factor and serologic blood group activity.

Highly active eluates from human milk[15] may also be inactivated by Clostridium enzyme. In one experiment the microbiologic activity of such preparation was reduced by 60 per cent of the original value after treatment with Clostridium enzyme for forty-eight hours.

TESTS WITH SALIVA ENZYME

All of a large number of samples of human saliva tested were found to be rich in Bifidus Factor.[1] Saliva of so-called secretors showed demonstrable serologic titer for blood groups A, B, or H, and of nonsecretors for Le^a.[1, 17] The presence of an enzyme in saliva, which destroys blood group activity, was first reported by Schiff.[2-6] We found in several experiments that incubation of saliva at 37° C. for forty-eight hours inactivates the Bifidus Factor as well as the serologic blood group characteristic present in saliva.

TABLE V. EFFECT OF ENZYME PREPARATION FROM Cl. welchii A.T.C.C. No. 7907 ON BLOOD GROUP AND BIFIDUS FACTOR ACTIVITY OF MUCOIDS

SUBSTRATE	INCUBATION TIME (HOURS)	BIFIDUS FACTOR ML./UNIT	BIFIDUS FACTOR DECREASE OF ACTIVITY (PER CENT)	SEROLOGIC TITER (RECIPR.) A OR B	SEROLOGIC TITER (RECIPR.) H
Meconium A or B, supernatant, dialyzed and boiled	0	0.3	-	512	128
	4	0.3	0	512	128
	16	0.3	0	512	128
	48	0.3	0	512	-
Ethanol (50-60 %) fraction of meconium, group A	0	0.49	-	8,000	-
	8	0.93	47	128	-
	48	3.1	84	64	-
Blood group B substance, (Sharp & Dohme)	0	0.39	-	512	128
	4	0.69	43.5	32	32
	16	1.1	64.5	<2	8
	48	3.1	87.5	<2	<2
Purified H substance from ovarian cyst, group O	0	0.22	-	-	32
	48	0.55	60	-	<1
Hog mucin (Wilson)	0	0.22	-	8,000	64
	48	2.0	89	8	<1

TABLE VI. DISTRIBUTION OF ENZYME, BLOOD GROUP SUBSTANCES AND BIFIDUS FACTOR IN SALIVA

SUBSTRATE	INCUBATION TIME (HOURS)	BIFIDUS FACTOR (ML./UNIT)	SEROLOGIC TITER (RECIPR.) A OR B	SEROLOGIC TITER (RECIPR.) H
Whole saliva	0	0.43	2,048	64
	48	inactive	4	1
Sediment	0	2.0	128	1
	48	4.0	1	1
Supernatant	0	0.75	1,024	64
	48	0.9	256	32

After centrifugation, the enzymatic activity of the saliva was found mainly in the sediment and the activity for Bifidus Factor and for blood group substances in the supernatant fluid (Table VI). To obtain more active enzyme preparations, the sediment was ground in a mortar, frozen, and suspended in 0.01 M phosphate buffer, pH 6.5, the volume of the preparation being adjusted to one-tenth that of the original saliva sample. Several substrates active for blood group and Bifidus Factor were tested with such saliva enzyme preparations. A set of observations, based on the average of two experiments, is con-

TABLE VII. INACTIVATION OF BLOOD GROUP SUBSTANCE AND OF BIFIDUS FACTOR BY SALIVA ENZYME

SUBSTRATE	INCUBATION TIME (HOURS)	BIFIDUS FACTOR ML./UNIT	BIFIDUS FACTOR DECREASE OF ACTIVITY (PER CENT)	SEROLOGIC TITER (RECIPR.) A	SEROLOGIC TITER (RECIPR.) H
Blood group substance A*	0	0.36	-	500,000	1,024
	48	0.32	-	32	1
Hog mucin	0	0.35	-	8,000	256
	48	0.32	-	64	4
Fraction from ovarian cyst	0	0.41	-	-	128
	48	0.60	32	-	4
Meconium (A), supernatant	0	0.24	-	2,048	16
	48	0.48	50	8	1
Eluate from human milk	0	0.23	-	-	-
	48	0.45	49	-	-

*Sharp & Dohme.

tained in Table VII. The reduction of the serologic blood group titer proceeded rapidly under the influence of the saliva enzyme and was practically complete after forty-eight hours. In contrast, only slight decrease in the activity of the Bifidus Factor was noticed in added substrates of human origin, and no destruction in substrates of animal origin.

TABLE VIII. INACTIVATION OF BLOOD GROUP SUBSTANCE AND BIFIDUS FACTOR BY SALIVA ENZYME WITH CONTINUOUS DIALYSIS

SUBSTRATE	ENZYME	BIFIDUS FACTOR MILLILITER PER UNIT	BIFIDUS FACTOR DECREASE OF ACTIVITY (PER CENT)	SEROLOGIC TITER (RECIPROCAL) A	SEROLOGIC TITER (RECIPROCAL) H
Blood group substance A*	Active	0.26	19	256	1
	Boiled	0.21	-	500,000	8,000
Hog mucin	Active	0.52	33	32	1
	Boiled	0.35	-	8,000	512
Fraction from ovarian cyst	Active	1.40	77	-	<1
	Boiled	0.33	-	-	128
Meconium (A), supernatant	Active	0.95	65	4	<1
	Boiled	0.33	-	1,024-2,048	8

Incubation time: forty-eight hours.
*Sharp & Dohme.

In another experiment, similar to the preceding, except that the mixture of substrate solution and enzyme was dialyzed against ten- to twentyfold volume of phosphate buffer during incubation, the destruction of serologic blood group activity was also approximately 100 per cent (Table VIII). The decrease in the activity of Bifidus Factor was found to be slightly greater than without dialysis, but was still very moderate with substrates of animal origin. A small, negligible percentage of the total activity of Bifidus Growth Factor was recovered in the dialysates of meconium and of the fraction of pseudomucinous ovarian cyst fluid after digestion with active saliva enzyme.

DISCUSSION

Unexpectedly, enzyme preparations obtained from *L. bifidus* var. *Penn* for which the Bifidus Factor is essential for growth will, under appropriate experi-

mental conditions, inactivate the required microbiologic nutrient. That release of sugars, particularly N-acetyl-D-hexosamine, from these high-molecular substances by enzymatic action is specifically related to loss of microbiologic activity has been substantiated by experiments with small, chemically well-defined compounds. β-methyl-N-acetyl-D-glucosaminide, which is very active as Bifidus Factor, is hydrolyzed by bifidus enzyme with release of N-acetyl-D-glucosamine and simultaneous decrease of microbiologic activity.[18] Such enzymatic inactivation coincident with hydrolysis was also observed with a disaccharide of N-acetyl-D-glucosamine and galactose prepared from hog mucin by Tomarelli and coworkers.[19] The enzymatic decomposition of this disaccharide proceeds with great velocity* indicating close affinity of the enzyme to the substrate. Bifidus Factor activity appears to be associated with a β-glycosidic linkage, since α-methyl-N-acetyl-D-glucosaminide is neither microbiologically active nor hydrolyzed by the bifidus enzyme preparation. It is of special interest that inactivation was not demonstrable when an enzyme preparation from a strain of *L. bifidus* which did not require the specific Bifidus Factor for growth was used.

No definite indication was found of a "depolymerizing enzyme" which would split high-molecular sources of Bifidus Factor into diffusible but still highly active components. It should be borne in mind, however, that the crude enzyme preparation used must contain a number of enzymes of which one may be a "depolymerizing enzyme," the reaction product of which is then further broken down and inactivated by other enzymes.

It was shown that reduction of "Bifidus Factor" activity by the "bifidus enzyme" is accompanied by reduction of serologic blood group activity where originally present. In general, the inactivation of the H-substance is easier and faster achieved than that of the A or B substance, in accordance with the greater lability of the H-substance.[16]

Clostridium enzyme, which is an ecto-enzyme, will also inactivate both "Bifidus Factor" and serologic blood group activity. The inactivation of blood group A and B activity by Clostridium enzyme appears to take place with greater speed than with the bifidus enzyme, whereas no such difference was noticed regarding the inactivation of the Bifidus Factor. Suspensions of crude meconium, even when boiled and dialyzed, are not inactivated in their blood group or Bifidus Factor activity by the Clostridium enzyme. From a teleologic point of view, this is of special interest, meconium being the first culture medium for bacteria in the intestinal lumen of newborn infants and Clostridia being among the first bacterial invaders, followed by *L. bifidus*. If the Bifidus Factor plays a part in establishing the Bifidus Flora in the newborn, its preservation during the first invasion of Clostridia fulfills a useful purpose. Apparently, this is made possible by the presence of an inhibitor in the crude meconium. Purified fractions of meconium with high activity for blood group substances or Bifidus Factor have lost the inhibitor and may be easily inactivated by Clostridium enzyme. It is equally noteworthy that saliva enzyme has a greater affinity to the Bifidus Factor as present in saliva than to added sources of Bifidus Factor of human and animal origin.

*Unpublished observations.

SUMMARY

Enzymes which inactivate both blood group activity and Bifidus Factor were demonstrated in a crude extract of *L. bifidus* var. *Penn* as well as in the culture filtrates of *Cl. welchii* Type B and in human saliva. No similar enzymes were found in a regular strain of *L. bifidus* which did not require the Bifidus Factor. Lactase, emulsin, amylase, and related enzymes were also inactive.

All specific sources of the Bifidus Factor, its high as well as low molecular forms, such as mucin, meconium, purified blood group substances, human milk, and purified fractions from human milk may be inactivated by the "bifidus enzyme." The inactivation is accompanied by the liberation of N-acetylhexosamine, fucose, and galactose and by reduction of serologic blood group activity.

The enzymes present in culture filtrates of *Cl. welchii* Type B, A.T.C.C. No. 7907, and in saliva exert a similar action. Crude meconium appears to contain an inhibitor which prevents inactivation by Clostridium enzyme but not by "bifidus enzyme" or by saliva. Purified fractions of meconium may be inactivated by Clostridium enzyme.

In saliva, the specific enzymatic activity is found, after centrifugation, in the sediment, whereas the Bifidus Factor and the blood group activity are present in the supernatant fluid. The saliva enzyme is very effective for the Bifidus Factor present in saliva, but its activity is weak for any added Bifidus Factor of human origin and negligible for Bifidus Factor of animal origin. Dialysis of the enzyme substrate mixture during incubation may enhance the inactivation of the Bifidus Factor by saliva enzyme. Blood group activity is always easily inactivated by saliva enzyme.

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CHROMATOGRAPHIC CHANGES IN PLASMA I^{131} DURING THE TREATMENT OF GRAVES' AND CARDIAC DISEASES CORRELATED WITH CLINICAL COURSE

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INTRODUCTION

THE determination of the dosage of I^{131} needed to obliterate thyroid function is still only a quasi-quantitative procedure. Attempts to compute dosages on estimates of thyroid weight and measurements of uptake and disappearance rate of a previous tracer (test) dose have not been entirely successful. Due to slow depletion of thyroxine stores in the gland a clinical evaluation of the effect of a therapeutic dose is subject to a delay of many weeks. A method for early estimation of the effect of thyroid function would save months of time in the clinical management of the patient. The identification of the iodinated components of plasma by paper chromatography led the authors to conceive the idea that alterations of these components during I^{131} therapy might be valuable in predicting therapeutic results.

These are readily identified by radioautography of simple one dimension filter paper chromatograms of the alcoholic extracts of blood plasma, a method which in the meantime has proved to be exceedingly useful in studying thyroid metabolism under various thyroid states.[1-9]

MATERIALS AND METHODS

Twenty-three patients receiving one or more therapeutic doses of I^{131} were studied. These cases were referred to this isotope unit for thyroid therapy due to clinical evidence of hyperthyroidism or cardiac disease with intractable angina pectoris. The diagnosis in each case was based on the usual classical signs and symptoms and laboratory criteria. The laboratory criteria also included radioiodine uptake over the thyroid and a blood I^{131} conversion procedure developed in this unit.[10-11] Nineteen patients had Graves' disease, and four patients had intractable angina pectoris.

The radioiodine† was given by mouth in dosage intended to give 9,000 r. to the thyroid according to Marinelli's formula.[12] The treatment was repeated after one to twelve months if the radiation effect was judged to be insufficient.

Samples of venous blood were withdrawn with a heparinized syringe at time intervals ranging between one to twenty-three days after the administration of isotope; the blood was centrifuged and the plasma withdrawn and 2.0 ml. were transferred to a standard dish for measurement of total I^{131} activity by use of scintillation counter.

Extraction Procedure.—The procedure devised for the extraction of the inorganic and organic I^{131} from plasma consisted of ethanol precipitation of the plasma protein and extraction of inorganic and organic I^{131}. Ten milliliters of absolute ethanol were added to 2.0 ml. of plasma in a 15.0 ml. graduated centrifuge tube. The tube was stoppered and shaken well by inversion for several minutes and centrifuged. The precipitate was washed

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†This radioiodine was purchased from Oak Ridge National Laboratories on authorization from the Isotope Division, United States Atomic Energy Commission.

with 5.0 ml. of absolute ethanol and the supernatants combined, the volume noted, and the I^{131} activity of a suitable aliquot determined. In vitro studies indicated that iodide tagged with I^{131} was completely extractable from plasma, and 60 to 80 per cent of added radiothyroxine was extractable by this procedure.

The ethanol extract (approximately 15.0 ml.) containing iodide and organic radioiodine was taken to dryness in a vacuum desiccator, and the residue was taken up in about 0.5 ml. of absolute alcohol and held for subsequent chromatographic studies.

Paper Chromatography.—A modification of the ascending one-dimensional technique of Williams and Kirby[13] was used. The plasma extract was applied to the paper in two fine lines (2 to 4 mm. wide, 9 cm. long, and 2 to 3 cm. apart) along a pencil line drawn 3 cm. above and parallel to the bottom of a square sheet of Schleicher and Schuell No. 598 filter paper (19 cm. × 19 cm.). This application was repeated in the presence of a current of air until the total volume (0.2 to 0.5 ml.) of extract was applied.

Reference standards of the following iodinated compounds were added to plasma and processed in the same manner: synthetic crystalline thyroxine, synthetic crystalline diiodotyrosine,* synthetic crystalline monoiodotyrosine, synthetic crystalline triiodothyronine, radioactive thyroxine and diiodotyrosine, radioactive monoiodotyrosine,* radioactive triiodothyronine,* and radioiodide with added carrier iodide prepared in this laboratory.

The R_F values of the above-mentioned compounds were determined during the development of this chromatographic method, but only iodide and an unknown substance at the solvent front were observed in the plasma after ingestion of I^{131}.

Each sheet of paper was attached to a glass lid with mounted paper clips, placed upright in a glass museum jar (10 inches high and 5 inches wide) containing a solvent mixture of 200 ml. of a N-propyl alcohol and 100 ml. of 0.5 N-NH_4OH covered with the closely fitting lid, and allowed to remain at room temperature for two to three hours at which time the solvent front (S.F.) has ascended about 15 to 16 cm. from the line of application (origin).

When the S.F. had ascended 15 to 16 cm. from the origin, the paper was removed, S.F. marked, and the paper dried in air. Papers containing added carrier standards were sprayed with a dilute solution of $NaNO_2$ and hydrochloric acid containing nitrite ion (NO_2) and the color developed by hanging the sprayed sheets in a glass museum jar saturated with ammonia vapor. An orange or red color† was produced with carrier triiodothyronine, thyroxine, diiodotyrosine, and monoiodotyrosine. This color slowly faded when taken from the container. R_F values for each compound were obtained by measuring the distance from the origin to the center of color or radioactivity band and dividing this distance by the measured distance between the origin and the S.F. The R_F values for the prepared iodinated compounds using N-propyl NH_4OH system were as follows: triiodothyronine 0.87-0.90, thyroxine 0.80-0.83, iodide 0.73-0.75, monoiodotyrosine 0.63-0.67, and diiodotyrosine 0.51-0.56. Each shipment of radiothyroxine was checked against crystalline thyroxine before use as a plasma reference source. The R_F value for crystalline thyroxine (color band) was indistinguishable from radiothyroxine and plasma radiothyroxine (radioautograph bands) following the administration of I^{131}.

Radioautographs.—After drying the processed paper, chromatograms containing I^{131} were placed against a sheet of "No-screen" x-ray film and mounted in an x-ray cassette (8 by 10 inches). Suitable film development for the plasma chromatograms containing I^{131} required from one to ten days' exposure. This time was predetermined by measuring the I^{131} activity at the origin with a Geiger tube and count rate meter just before chromatographic development.

RESULTS

Radioautographs of chromatograms of plasma over several days after the administration of I^{131} are shown in Figs. 1 and 2. The first is typical of the

*Samples of these compounds were supplied through the courtesy of Dr. Kenneth G. Scott.[14]

†At least 3 to 5 μg of the compound per square centimeter of paper was necessary for color development.

class failing to respond to I^{131}. The second is typical of the cases that afterward went on to a hypothyroid state. The latter patient is one of four who are still taking thyroid substance up to one and one-half years after treatment.

The results for all twenty-three cases are set forth in Tables I, II, III, IV, and V. They have been grouped as follows:

Group I—Table I.—Five hyperthyroid cases responded to the first therapeutic dose of I^{131}. Their plasma chromatograms all showed marked reduction in the thyroxine band within a few days after the administration of I^{131}.

Group II—Table II.—Five hyperthyroid patients required two doses of I^{131} before a satisfactory response was obtained. All showed good thyroxine bands for at least three days after the first dose of I^{131}, some as long as eight days, one for twenty-three days. All cases in this group showed a marked reduction of the thyroxine band after a second dose, which was given a few weeks to some months after the first, and all showed good remission of hyperthyroidism following the second therapeutic dose.

Group III—Table III.—Seven hyperthyroid patients did not respond well to I^{131} (none of these has yet received a second dose). The chromatograms of this group were the same as those observed in Group II—Table II following the first dose of I^{131}.

Group IV—Table IV.—Two hyperthyroid patients did not respond well to second dose of I^{131}. The chromatograms of this group were the same as in Group III—Table III.

Group V—Table V.—Five cardiac cases composed this group. None of these was hyperthyroid, and none has so far turned hypothyroid or shown any improvement in cardiac reserve. All but one were given two doses. The chromatograms of this group indicated that there was a gradual turnover of iodide to thyroxine with little or no change in the formed thyroxine band.

An I^{131}-tagged substance which was not iodide, thyroxine, triiodothyronine, monoiodotyrosine, or diiodotyrosine was observed and was most marked in samples from those patients who later became hypothyroid. The relative amounts bound increased with time after I^{131} was administered.

Four cases developed signs and symptoms of hypothyroidism. Two were in Group I and two in Group II. The plasma chromatograms of all of these show complete or almost complete disappearance of the thyroxine band within two to eight days.

DISCUSSION

An approximate evaluation of the effectiveness of I^{131} therapy appears possible within a week after a therapeutic dose by observing the evolution of the thyroxine band in the radioautograph of the filter paper chromatogram of the blood plasma.

Disappearance of this thyroxine band by two to seven days suggests that the patient will end up markedly hypothyroid.

Concurrently with the disappearance of thyroxine there appears an increasing I^{131} activity associated with an unknown substance at the solvent front. This is believed to be an iodinated lipid. The presence of a lipidlike substance

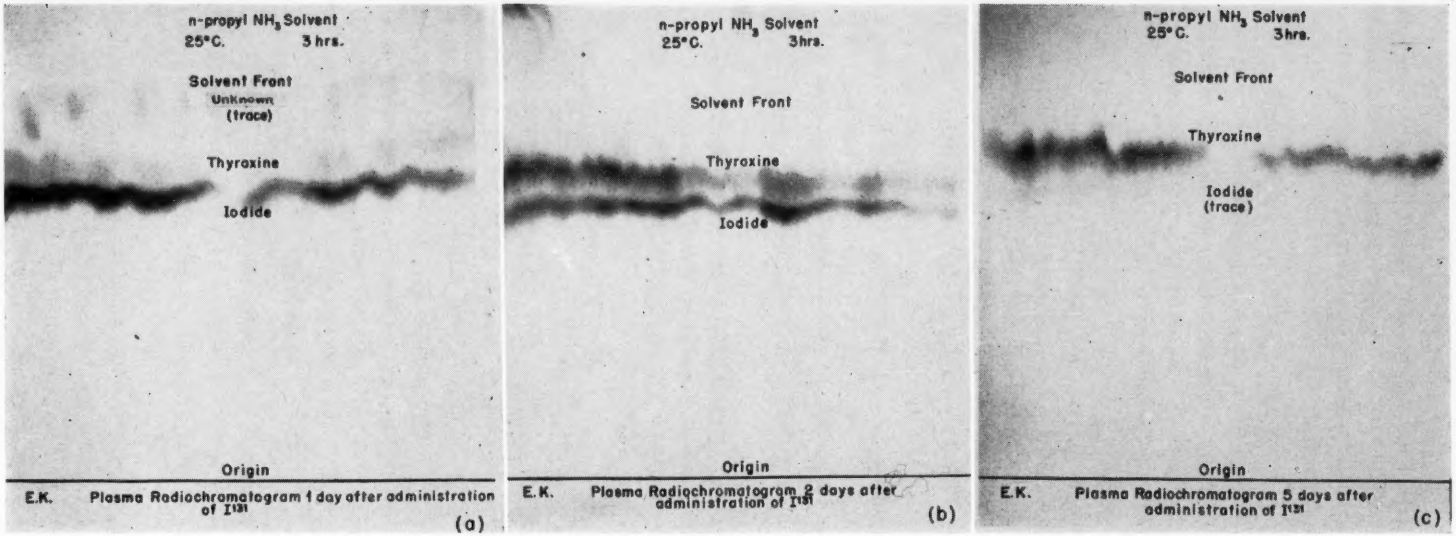

Fig. 1.—Case typical of failure to respond to therapy. Radioautograms of filter paper chromatograms of plasma extract from hyperthyroid patient after ingestion of I^{131}. *a*, Note strong iodide component, weak thyroxine component and trace of weak unknown substance. *b*, Observe the increase in thyroxine component with decrease in iodide component and absence of unknown substance. *c*, Note stable thyroxine component, trace of iodide, and absence of unknown substance. (See Table V, Case 4.)

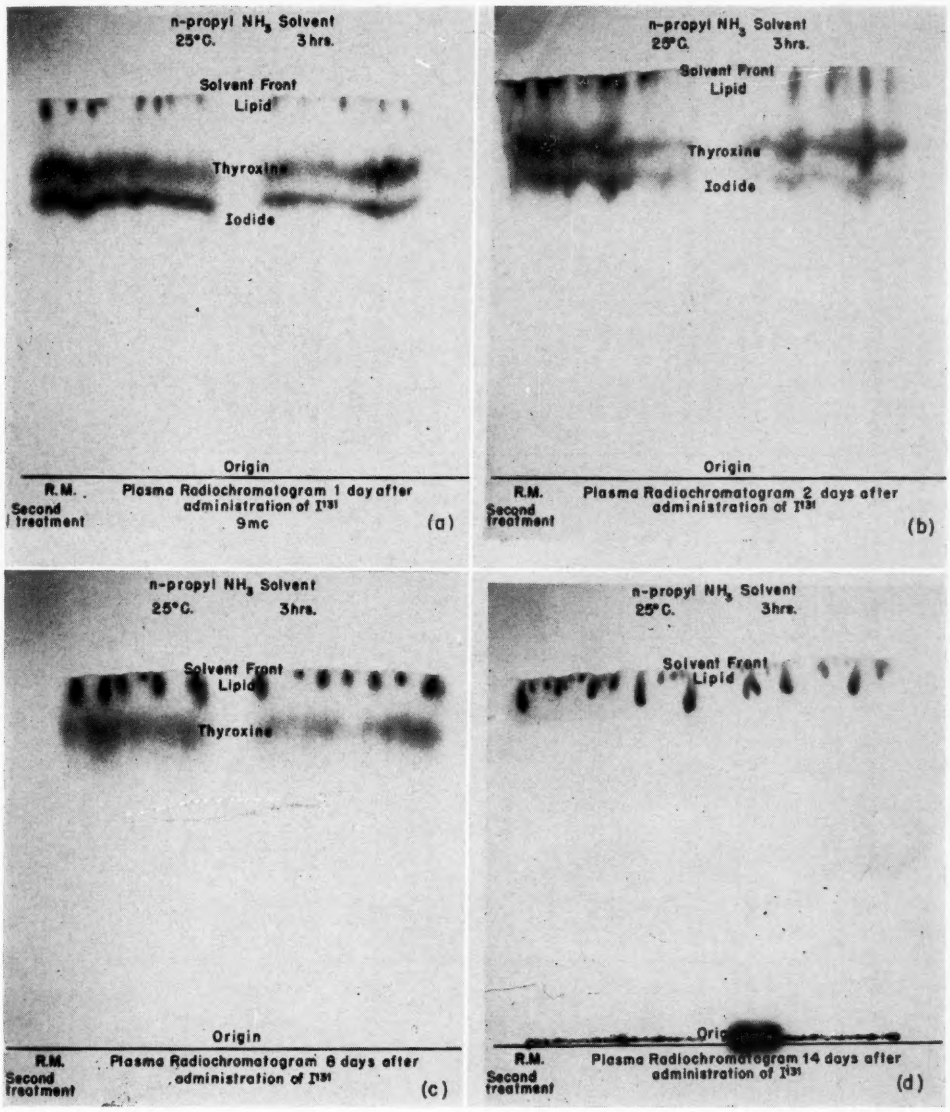

Fig. 2.—Case typical of strong response to therapy. Radioautograms of filter paper chromatograms of plasma extract from a hyperthyroid patient after ingestion of I^{131}. *a*, Note strong components of thyroxine and iodide and the appearance of an unknown substance. *b*, Note little or no change in iodide and thyroxine components with an increased concentration of unknown substance. *c*, Note the absence of the iodide component with a further increase in unknown substance. *d*, Note that only the unknown substance remains. (See Table II, Case 1.)

TABLE I. HYPERTHYROID CASES RESPONDING TO FIRST DOSE OF I^{131}

PATIENT AGE DIAGNOSIS	DOSE BY MOUTH DATE	I^{131} UPTAKE 24 HRS.	PLASMA PARTITION RADIOAUTOGRAMS DAYS AFTER DOSE	IODINATED COMPONENTS IODIDE	THYROXINE	? SUBSTANCE*	FOLLOW-UP	CLINICAL STATUS
GROUP I								
1—R. W.	7.5 mc.	53%	1	+++	0	0	2½ mos. 30% uptake in 24 h.	Definite improvement
31 yrs.	5 Nov. 51		2	+	++	+	4 mos. 11% uptake in 24 h.	Further improvement
Diffuse toxic goiter			3	++	++	++	6 mos.	Euthyroid
(recurrent rt. lobe)			4	+	+	+++	7 mos.	Mild hypothyroid
			Note: Reciprocal relationship between iodide and thyroxine not maintained; lowered production thyroxine with definite increase in unknown substance				9mos.	On thyroid therapy
							10 mos. 14% uptake in 24 h.	Off thyroid; euthyroid
							14 mos. 11% uptake in 24 h.	Euthyroid
							Note: effectiveness of single dose	
2—L. G.	5.1 mc.	76%	1	+	++	++	2 mos. 0% uptake in 24 h.	Hypothyroid
31 yrs.	24 May 51		2	0	0	+++	4 mos.	Thyroid replacement therapy
Diffuse toxic goiter			Note: Rapid loss of thyroxine and iodide leaving only elevated unknown substance				11 mos.	Therapy continued
							Note rapid effect of treatment	
3—S. J.	6.7 mc.	68%	1	+	++	trace	2¾ mos. 6% uptake in 24 h.	Mild hypothyroid
31 yrs.	20 Sept. 51		3	0	+++	+	14½ mos.	Hypothyroid; thyroid replacement therapy
Diffuse toxic goiter			6	+	trace	++		
			Note: Definite decrease in thyroxine with appearance of moderate unknown substance				Note effectiveness of treatment	
4—E. C.	5.0 mc.	51%	1	trace	++	trace	4 mos. 34% uptake in 24 h.	Euthyroid
65 yrs.	22 Oct. 51		2	+	+	+	8 mos. 35% uptake in 24 h.	Euthyroid
Toxic nodular goiter			4	+	+	++	14 mos. 30% uptake in 24 h.	Euthyroid
			Note: Lowered production of thyroxine with increasing unknown substance					
5—W. M.	8.8 mc.	76%	2	++	+	++	3¼ mos. 3% uptake in 24 h.	Euthyroid
43 yrs.	12 Aug. 52		3	+	+	+++		
Diffuse toxic goiter			Note: Lowered production of thyroxine with elevated unknown substance				Note effectiveness of treatment	

*Activity gathered at solvent front in chromatograms.

TABLE II. HYPERTHYROID PATIENTS REQUIRING TWO DOSES OF I^{131} BEFORE SATISFACTORY RESPONSE

PATIENT AGE DIAGNOSIS	DOSE BY MOUTH DATE	I^{131} UPTAKE 24 HRS.	PLASMA PARTITION RADIOAUTOGRAMS: DAYS AFTER DOSE	IODINATED COMPONENTS: IODIDE	THYROXINE	? SUBSTANCE*	FOLLOW-UP	CLINICAL STATUS
GROUP II								
1—R. M.	4.8 mc.	62%	1	+	++	0	2½ mos. 38% uptake in 24 h.	Little or no improvement; still hyperthyroid
56 yrs.	21 Sept. 51		3	0	+++	0		
Diffuse toxic goiter			Note: Reciprocal relationship between iodide and thyroxine with no unknown substance					
	9.0 mc.	36%	1	+++	+++	+	2½ mos. 3% uptake in 24 h.	Complete remission
	12 Dec. 51		2	+++	+++	++		Eventually needed thyroid replacement therapy
			3	trace	+++	++		
			4	trace	+++	++		
			6	trace	+++	+++		
			8	0	++	+++	Note effectiveness of second dose	
			9	0	+	+++		
			17	trace	0	+++		
			21	0	0	+++		
			Note: Lowered rate of conversion of iodide to thyroxine; gradual disappearance of thyroxine with increasing unknown substance					
2—C. W.	6.0 mc.	70%	1	++	++	+	3 mos. 43% uptake in 24 h.	Little or no change; still hyperthyroid
38 yrs.	31 Oct. 51		5	0	+++	trace	6 mos. 56% uptake in 24 h.	Still hyperthyroid
Diffuse toxic goiter			Note: Reciprocal relationship between iodide and thyroxine (rapid conversion) with slight unknown substance					
	4.0 mc	56%	1	+++	+	++	2 mos. 3% uptake in 24 h.	Mild hypothyroid euthyroid
	4 Jun. 52		2	+	+	+++	Note effectiveness of second dose	Eventually needed thyroid replacement therapy
			Note: Lowered production of thyroxine with elevation of unknown substance					
3—G. W.	10.6 mc.	48%	1	++	++	trace	3 mos.	Hyperthyroid
32 yrs.	26 Feb. 52		2	+	+++	0		
			3	trace	+++	0		
Diffuse toxic goiter			Note: Reciprocal relationship between iodide and thyroxine; no unknown substance					
	8.0 mc.	50%	1	+++	+	0	2½ mos.	Euthyroid
	24 May 52		6	++	++	++	10 mos. 24% uptake in 24 h.	Euthyroid
			Note: Lowered conversion of iodide to thyroxine with moderate unknown substance				Note effectiveness of second treatment	

TABLE III. HYPERTHYROID PATIENTS NOT RESPONDING WELL TO FIRST DOSE OF I^{131} BUT NOT GIVEN SECOND DOSE

PATIENT AGE DIAGNOSIS	DOSE BY MOUTH DATE	I^{131} UPTAKE 24 HRS.	PLASMA PARTITION RADIOAUTOGRAMS				FOLLOW-UP	CLINICAL STATUS
			DAYS AFTER DOSE	IODINATED COMPONENTS				
				IODIDE	THYROXINE	? SUBSTANCE*		
GROUP III								
1—J. T. 61 yrs. Diffuse toxic goiter	6.3 mc. 10 Feb. 53	55%	1	+++	+++	+	1½mos. 64% uptake in 24 h.	Hyperthyroid
			2	+	+++	trace		
			3	+	+++	0		
			Note: Reciprocal relationship between iodide and thyroxine with little or no unknown substance					
2—T. G. 28 yrs. Diffuse toxic goiter	7.0 mc. 25 Aug. 52	54%	1	+++†	+	0	3 mos.	Hyperthyroid
			2	++	++	0	6 mos.	Hyperthyroid
			3	++	++	0		
			4	++	+++	trace		
			12	+	+++	+		Died, March '53, of pleural pneumonia
			Note: Maintained strong thyroxine with gradual drop in iodide; low concentration of unknown substance					
3—Q. W. 27 yrs. Diffuse toxic goiter	10.0 mc. 8 May 51	67%	1	++	+++	+	3 mos. 10% uptake in 24 h.	Definite improvement, incomplete remission
			6	+	+++	0		
			15	0	+++	+	19 mos. 25% uptake in 24 h.	Incomplete remission
			23	trace	++	+	25 mos. 40% uptake in 24 h.	Incomplete remission
			Note: Reciprocal relationship between iodide and thyroxine with low concentration of unknown substance					
4—R. A. 24 yrs. Diffuse toxic goiter	5.2 mc. 26 Jun. 51	62%	1	+++	+	0	3 mos. 5% uptake in 24 h.	Some change; still hyperthyroid
			2	+	+++	0		
			3	+	+++	0	6 mos. 30% uptake in 24 h.	Hyperthyroid
			4	+	++	trace		
			Note: Reciprocal relationship between iodide and thyroxine with no unknown substance					

4—L. S. 56 yrs. Diffuse toxic goiter	12.0 mc. 23 Apr. 51	35%	1 10	trace 0	+++ +++	0 0	$4\frac{1}{2}$ mos. 35% uptake in 24 h. $5\frac{1}{2}$ mos. 48% uptake in 24 h.	Hyperthyroid Hyperthyroid
			Note: Extreme rate of conversion of iodide to thyroxine (only trace of iodide present at 24 hrs.); no unknown substance					
	10.2 mc. 25 Oct. 51	45%	1 2 4	trace 0 0	+++ ++ 0	+ ++ +++	$2\frac{1}{2}$ mos. 10 mos. 20% uptake in 24 h.	Euthyroid Euthyroid
			Note: Disappearance of thyroxine with increasing unknown substance					
5—M. H. 37 yrs. Diffuse toxic goiter with Hypertensive Cardiovascular Disease	7.1 mc. 14 Feb. 52	68%	1 2 3 4	+++ ++ + +	+ ++ +++ +++	0 0 0 trace	1 mo.	Hyperthyroid
			Note: Reciprocal relationship between iodide and thyroxine with no unknown substance					
	4.85 mc. 3 Mar. 52	24%	1 2 3 4	+++ ++ ++ +	0 + ++ ++	0 ++ ++ ++	3 mos. 18% uptake in 24 h. 7 mos. 40% uptake in 24 h.	Euthyroid. Euthyroid.
			Note: Lowered production of thyroxine with moderate unknown substance				Note moderate effect	
	4.5 mc. 29 Oct. 52	33%	1 2 5 6	+++ +++ ++ ++	0 0 + ·	++ ++ ++ ++	4 mos. 9% uptake in 24 h. Note: 3rd treatment dose given due to Hypertensive Cardiovascular Disease	Mild hypothyroid Note effectiveness of 3rd treatment in obtaining desired hypofunction due to cardiac condition
			Note: Minimum level of thyroxine production remaining					

*Activity gathered at solvent front in chromatograms.

TABLE IV. HYPERTHYROID PATIENTS NOT RESPONDING WELL TO TWO DOSES OF I^{131}

PATIENT AGE DIAGNOSIS	DOSE BY MOUTH DATE	I^{131} UPTAKE 24 HRS.	PLASMA PARTITION RADIOAUTOGRAMS				FOLLOW-UP	CLINICAL STATUS
			DAYS AFTER DOSE	IODINATED COMPONENTS				
				IODIDE	THYROXINE	? SUBSTANCE*		
GROUP IV								
1—W. C.	9.1 mc.	62%	1	+	++	0	$2\frac{1}{2}$ mos. 65% uptake in 24 h.	Hyperthyroid (no change)
32 yrs.	23 Apr. 51		7	0	+++	0		
Diffuse toxic goiter			Note: Reciprocal relationship between iodide and thyroxine with no unknown substance					
	5.0 mc.	76%	1	+	++	trace	2 mos. 7% uptake in 24 h.	Some improvement, remission incomplete
	13 Jul. 51		4	0	+++	+		
			Note: Reciprocal relationship between iodide and thyroxine with some unknown substance				3 mos.	Still hyperthyroid
							5 mos. 40% uptake in 24 h.	Still hyperthyroid
2—C. W.	4.5 mc.	73%	5	++	+++	+	1 mo.	Hyperthyroid
54 yrs.	2 Oct. 52		7	++	+++	trace		
Diffuse toxic goiter			Note: Maintained strong thyroxine with small amount of unknown substance					
	5.0 mc.	40%	3	+++	++	++	4 mos. 30% uptake in 24 h.	Euthyroid
	14 Nov. 52		4	++	+++	++	7 mos. 42% uptake in 24 h.	Mild hyperthyroid
			Note: Only slight change in iodinated components compared to first treatment					

*Activity gathered at solvent front in chromatograms.

5—L. D. 29 yrs. Diffuse toxic goiter Rt. lobe recurrence after surgery	3.1 mc. 10 Sept. 51	63%	1	+	++	0	8 mos. 58% uptake in 24 h.	No change
			2	0	+++	0	11 mos. 50% uptake in 24 h.	Slight improvement; still hyperthyroid
			Note: Reciprocal relationship between iodide and thyroxine (rapid conversion), with no unknown substance				15 mos. 36% uptake in 24 h.	Hyperthyroid
							21 mos. 42% uptake in 24 h.	Hyperthyroid
6—J. S. 59 yrs. Diffuse toxic goiter with abdominal carcinoma	7.0 mc. 4 Apr. 52	68%	1	++†	+	trace	4 mos.	Some improvement; remission incomplete
			3	++	++	0		
			4	++	++	trace	5 mos.	Died of extensive abdominal Ca.
			5	++	++	++		
			6	++	+++	++		
			8	+	+++	trace		
			Note: Maintained iodide and strong thyroxine with some unknown substance					
7—A. J. 69 yrs. Toxic adenoma with H.C.V.D.	5.3 mc. 1 Nov. 51	50%	4	+	++	+	4 mos. 34% uptake in 24 h.	Some change; still hyperthyroid.
			5	trace	+++	++		
			6	0	+++	++		
			7	0	+++	+		
			Note: Reciprocal relationship between iodide and thyroxine with some unknown substance					

*Activity gathered at solvent front in chromatograms.
†On anti-thyroid medication during I^{131} therapy.

TABLE V. CARDIAC PATIENTS NOT RESPONDING TO ONE OR TWO DOSES OF I^{131}

PATIENT AGE DIAGNOSIS	DOSE BY MOUTH DATE	I^{131} UPTAKE 24 HRS.	PLASMA PARTITION RADIOAUTOGRAMS DAYS AFTER DOSE	IODINATED COMPONENTS IODIDE	THYROXINE	? SUBSTANCE*	FOLLOW-UP	CLINICAL STATUS
GROUP V								
1—G. L.	7.0 mc.	45%†	1	+++	0	+	1½ mos. 10% uptake in 24 h.	No change in cardiac condition
54 yrs.	21 Mar. 52		3	++	+	trace		No signs or symptoms of hypothyroid function
			4	++	++	0		
			7	++	++	0		
Angina pectoris			Note: Gradual shift of iodide to thyroxine with little or no unknown substance					
Normal thyroid gland	9.2 mc.	10%	1	+++	0	0	4 mos. 30% uptake in 24 h.	Same as above
	20 May 52		3	++	+	0		
			Note: Same pattern as first treatment					
2—G. K.	10.0 mc.	46%†	2	+++	+	0	2 mos.	No change in cardiac condition
61 yrs.	23 May 52		4	++	++	0		No signs or symptoms of hypothyroid function
Angina pectoris			Note: Gradual shift of iodide to thyroxine with no unknown substance					
Normal thyroid gland								
3—J. C.	5.1 mc.	38%	1	+++	0	trace	1 mo. 25% uptake in 24 h.	No signs or symptoms of hypothyroid function
61 yrs.	12 Feb. 53		2	+++	0	0		
Angina pectoris			5	+	++	0		No change in cardiac condition
Normal			Note: Delayed turnover of iodide to thyroxine with little or no unknown substance					
thyroid gland	9.5 mc.	25%	1	+++	0	trace	2½ mos. 24% uptake in 24 h.	Same as above
	12 Mar. 53		2	+++	0	0		
	10.3 mc.	25%	Note: Delayed turnover pattern still present with little or no unknown substance					
	8 Jun. 53							
4—E. K.	7.0 mc.	35%	1	+++	+	trace	3 mos. 40% uptake in 24 h.	No change in cardiac condition
60 yrs.	30 Apr. 52		2	+++	++	0		No signs or symptoms of hypothyroid function
Angina pectoris			5	trace	+++	0		
Normal			7	trace	+++	0		
thyroid gland			8	0	+++	trace		
			9	trace	++	+	8 mos. 36% uptake in 24 h.	Same as above
			Note: Reciprocal relationship between iodide and thyroxine with little or no unknown substance					
	8.0 mc.	35%	3	trace	+++	0	3 mos. 8% uptake in 24 h.	Same as above
	28 Jan. 53		Note: Reciprocal relationship maintained with no unknown substance				4 mos. 16% uptake in 24 h.	Same as above

*Activity gathered at solvent front in chromatograms.

†These elevated uptakes were produced by terminating propylthiouracil therapy one week prior to I^{131} therapy.

at R_F 0.95 in plasma chromatograms was also reported by Rosenberg.[3] Empirically this seems to be a worth-while addition to the chromatographic evidence of destruction of thyroid function. More work will be carried out to identify this unknown substance chemically.

Chromatographic plasma analysis may provide the means for obtaining closer control of I^{131} therapy of Graves' disease. Within two to seven days the evidence obtained appears to indicate that one can predict the therapeutic result which clinically is not evident for one to five months because of the slow depletion of thyroxine stores in the gland.

SUMMARY

Filter paper chromatographs were made of the blood plasma of 19 hyperthyroid patients and 4 patients with intractable angina pectoris. Plasma radioautographs show correlation as follows: disappearance of the thyroxine band usually between two to seven days forecasts clinical hypothyroidism, whereas reduced intensity of the band during this same interval forecasts euthyroidism.

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THE METABOLISM OF C^{14} LABELED ETHYLENEDIAMINETETRAACETIC ACID IN HUMAN BEINGS

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ETHYLENEDIAMINETETRAACETIC acid is a recently developed therapeutic agent used in the treatment of lead[1, 2] and radioactive metal poisoning,[3] as an anticoagulant in blood used for transfusion,[4] and in other instances where control and investigation of metal ion concentration is desired.[5] A study of the metabolism of the compound was undertaken to gain information which might be used for more effective application of the material, in particular for establishing optimum dosage schedules, and to reveal any possible deleterious effects which might result from prolonged use of the drug. The calcium chelate of the compound was studied since this is the form in which the drug is most commonly employed.

METHODS

Young adult healthy men were used as subjects. Studies were carried out after intravenous, intramuscular, and oral administration and after application on the skin. Three subjects were used in each group.

In the intravenous studies, the injection solution was prepared by mixing 2.2 mg. of Ca ethylenediamine-2-C_4^{14} acetic acid, yielding approximately 2.5×10^7 disintegrations per minute (d/m) with 2 Gm. of "cold" (unlabeled) $CaNa_2$ EDTA* in a total of 10.5 ml. of water solution. The C^{14} labeled material was prepared by Murray and Ronzio.[6] The "cold" $CaNa_2$ EDTA was prepared as a 20 per cent neutral solution in water, especially for drug use. The injections were given over two- to three-minute periods into the antecubital arm vein. Blood samples were drawn at appropriate intervals indicated in Fig. 1, and in one case a spinal fluid sample was obtained. Urine was collected as voided for twenty-four hours, at which time the activity had fallen below detection levels. In two of the intravenous studies blood pressure readings and four-lead electrocardiogram tracings were made every fifteen minutes starting one-half hour before the administration of dose and continuing for an hour after. This was done to determine any cardiovascular changes brought about by the drug.

The dose for intramuscular studies consisted of 2.2 mg. C^{14} labeled Ca EDTA mixed with 1 Gm. "cold" Ca EDTA and 2 ml. of a 1 per cent procaine solution, all made up to 10 ml. with water. Injections were made into the buttocks, 5 ml. on each side. Blood and urine samples were collected as in the intravenous studies. Feces were not collected since pilot studies indicated 85 to 98 per cent of the activity could be recovered in the urine.

For oral administration, 1.5 mg. of C^{14} labeled Ca EDTA yielding approximately 1.6×10^7 d/m was dissolved in one-half ml. of water and given in a gelatin capsule. Urine and feces were collected for assay. Blood samples were drawn in the first study but it was found that the level of activity was too low to measure and hence this was not done in subsequent studies.

The dose used in skin absorption studies was prepared in a thin paste of Aquaphor containing 2.0 mg. C^{14} labeled Ca EDTA yielding 2.2×10^7 d/m and 1 Gm. of "cold" Ca EDTA. In one study KY jelly was the water-soluble base and Na EDTA was used in the

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place of Ca EDTA. The paste containing the dose was spread over an area of 100 cm.^{2} on a sheet of thin latex and taped firmly to the lateral chest wall just inferior to the axilla. It was kept there for twenty-four hours. The activity appearing in the urine was used as the measure of the absorption which had taken place.

Assays were carried out by plating the samples on stainless steel circular disks having a surface area of 15 cm.^{2} and counting in a windowless, methane-argon gas flow proportional counter. Most urine samples were plated directly since the activity was sufficiently high that small aliquots, i.e., 100 to 500 microliters gave satisfactory counts. In the oral studies, however, it was necessary to concentrate the urine by evaporation before plating. In these samples, mass absorption corrections were necessary. Because of the very low activity, the urine from skin absorption studies required special treatment. To the urine, which was made slightly alkaline, was added 100 to 200 mg. of "cold" Na EDTA as carrier. The sample was then brought to pH 1-2 with a methanol hydrochloric acid solution and the entire EDTA fraction, including the tracer, was precipitated out. The precipitate was separated by centrifugation and dissolved in 1 ml. of concentrated NH_4OH. An aliquot of this solution was plated and counted.

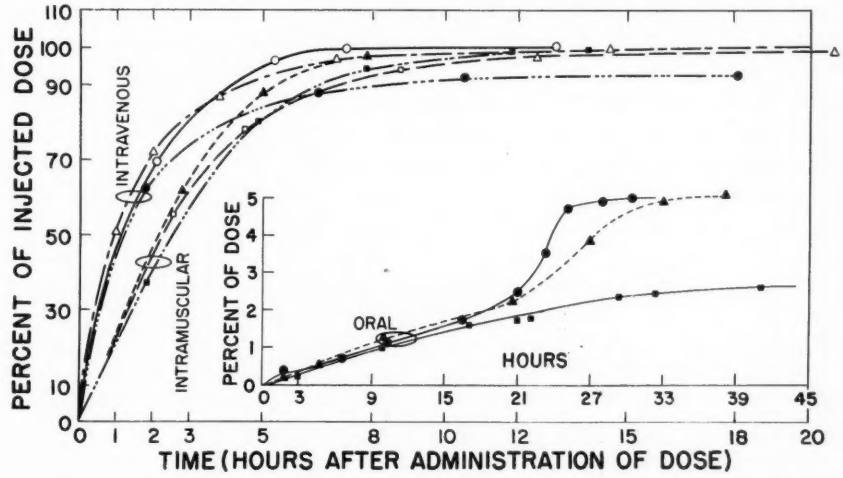

Fig. 1.—Cumulative urinary excretion after administration of C^{14} Ca EDTA.

Feces and blood samples required special processing before assaying. The active material in the feces was removed by repeated extraction with 1.5 per cent NH_4OH. The extraction solutions were concentrated to a small volume, 5 to 10 ml., by evaporation and an aliquot taken of this for assay. Mass absorption corrections were necessary. In some of the samples the concentration of the extraction fluid resulted in the formation of a floc of protein which had been carried over during the NH_4OH extraction. In these instances 5 ml. of 5 per cent zinc sulfate solution was added to insure complete precipitation and the precipitate was separated by centrifugation. It was necessary to keep the solution alkaline with Na_2CO_3 to prevent loss of tracer via the precipitate.

At first, assays were carried out on both whole blood and plasma, principally to determine the partition of the activity between the two compartments. In subsequent studies, after this ratio had been established, assays were run only on the plasma. Assays were carried out on protein-free filtrates. Protein-free filtrates were prepared by the barium hydroxide-zinc sulfate technique. Five milliliters of a saturated phenol solution were added to insure complete precipitation. The protein precipitate was washed with a 1.5 per cent NH_4OH solution to remove last traces of activity. The filtrate and washes were concentrated by evaporation and a suitable aliquot was taken for counting.

The identification of the active material in the urine was done by filter paper chromatography. The method of Consden and co-workers[7] was followed with slight modification. Aliquots of 0.1 ml. of urine yielding approximately 6×10^4 d/m were used. The chromatograms were developed for twelve to fifteen hours in a descending column at approximately 25° C. with a mixture of 1:1 isopropyl alcohol and 0.2 N HCl. The final location of the activity on the strip were determined by the use of autoradiographs.

RESULTS

Except when urine or feces samples were missed, almost all of the parenterally-administered material could be accounted for in the urine within twenty-four hours. In the oral studies it was two to three days before all of the dose appeared in the excreta. The average recovery in the intravenous studies was 98.1 ± 4.6 per cent. Following intramuscular injection 98.8 ± 1.6 per cent was used in the urine and after oral administration an average of 91 ± 2.0 per cent appeared in the feces and 4.2 ± 2.0 per cent in the urine. Cumulative urinary excretion plotted against time is presented in Fig. 1. Fifty per cent of the dose was excreted by one hour after intravenous injection and by two and a half hours after intramuscular injection. By seven hours at least 90 per cent of the dose was excreted after either mode of parenteral administration. The material continued to be absorbed for at least eighteen hours after oral administration, although the total amount was low: a maximum of 5 per cent, as judged by the amount appearing in the urine. Excretion continued in the feces for two to three days. Table I presents the cumulative fecal excretion.

TABLE I. CUMULATIVE FECAL EXCRETION AFTER ORAL ADMINISTRATION, EXPRESSED IN PERCENTAGE OF ADMINISTERED DOSE

TIME (HOURS)	SUBJECT		
	I	II	III
0-24	48.3	57.2	60.0
24-48	77.0	83.7	92.8
48-72	89.6	90.0	93.3

The maximum of activity in the urine after application of the drug to the skin was 0.001 per cent, indicating a very low level of absorption.

The distribution of activity between cells and plasma was determined by separate assays of whole blood and plasma on several of the blood samples and calculated from the following relationship:

$$\% \, A_{rc} = \frac{A_{wb} - A_p \, (1\text{-hematocrit})}{A_{wb}} \times 100,$$

where A_{rc}, A_{wb}, and A_p represent the activity in the red cell fraction, the activity per milliliter of whole blood and the activity per milliliter of plasma, respectively. The hematocrit is expressed as a fraction. Direct determination of activity in the separated red cell fraction was undesirable because of the possible loss of activity during the washing process. As can be seen from Table II the activity resides principally, if not entirely, in the plasma. The small deviations in column four are probably due to experimental uncertainties.

TABLE II. PARTITION OF ACTIVITY BETWEEN RED CELLS AND PLASMA

		TIME AFTER ADMINISTRATION (HOURS)	A_{wb}* C./MIN./ML.	A_p† C./MIN./ML.	PER CENT A_{rc}‡
Subject I		½	660	1,360	− 1.2
		1	444	814	+11.0
Hematocrit	.509	1½	333	663	+ 2.3
Subject II		½	430	810	+ 3.2
		1	222	483	−12.0
Hematocrit	.485	1½	160	340	− 9.4

*A_{wb} = Activity per ml. of whole blood.
†A_p = Activity per ml. of plasma.
‡% A_{rc} = Per cent activity in the red cell fraction.

The disappearance of the activity from the blood is illustrated in the blood clearance curves in Fig. 2. The half time for clearance was one hour and five minutes for the intravenous study, and one and one half hours after intramuscular administration. The level of activity in the blood never reached a level sufficiently high to be detected after oral administration or after application to the skin.

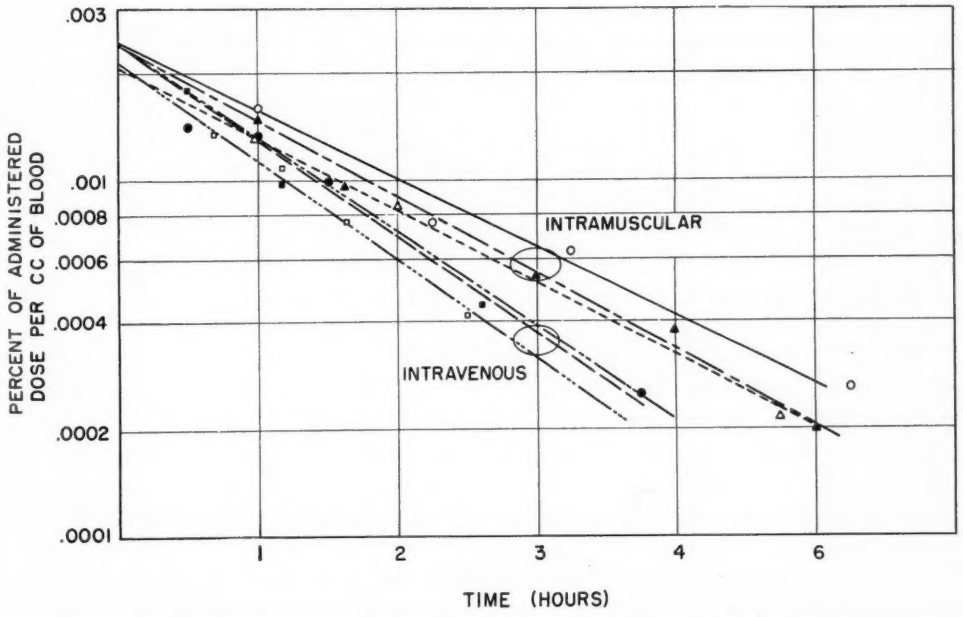

Fig. 2.—Blood disappearance curves following administration of C^{14} labeled Ca EDTA.

In the study in which the transfer across the blood spinal fluid barrier was measured, it was found that the level of activity in the spinal fluid was approximately one-twentieth that of the plasma, namely 45 c./m./ml. in the cerebrospinal fluid as compared to 870 c./m./ml. of plasma. The measurements were made one hour after intravenous injection of the dose. In the two

studies in which blood pressure, pulse, and electrocardiograms were followed before and after intravenous injection of the drug, no unusual cardiovascular changes could be detected.

The chromatographed urine samples yielded a single band of activity with an R_F value of 0.8. This corresponded to the band which was identified to be EDTA in urine obtained from rats subjected to a similar metabolic study.[8]

DISCUSSION

The appearance of over 95 per cent of the injected activity in the urine and the identification of this activity as labeled Ca EDTA indicate that the metabolism of the compound is singularly uncomplicated; namely, that it passes through the body apparently unaltered and is excreted almost entirely through the kidney. It appears that the only interaction in the body is combination with certain di- and tetravalent metal ions and that any therapeutic or deleterious effects occur solely via this mechanism. Because of the rapid and complete elimination of the drug, the only likely long term deleterious effect from prolonged administration one might consider, is a slow depletion of the body of an essential ion, perhaps iron, cobalt, or manganese, etc. This possibility is not answerable by the present study.

Consideration of the blood clearance curves shows that shortly after parenteral administration the drug leaves the blood and mixes with a large volume of tissue fluid, namely from 40.8 to 47.6 liters* in the various individuals studied. These figures represent from 58.2 to 68 per cent of the body weights of the subjects, which is approximately the entire body water. The material, however, must not mix with all of body water initially since, as seen from Table II, it does not enter the red cells, and, as seen from the spinal fluid study, it passes very slowly across the blood-spinal fluid barrier.

Renal clearance, determined from the equation $C = \frac{0.693}{T\ 1/2} V$, where C = renal clearance, T 1/2 = half time of blood clearance, and V = the dilution volume (see Foreman, Vier, and Magee[8]), gives a value for the intravenous study of 680 ml. per minute which indicates the material is cleared by tubular excretion as well as by glomerular filtration. The rapid turnover time suggests that frequently repeated doses will be required to maintain blood levels, unless some device for slow continuous release of the material into the blood stream is used.

The low absorption after oral administration is very surprising in view of the finding that the material is effective by that route in accelerating the excretion of yttrium[2] and lead.[1] There is no satisfactory readily apparent explanation of this at present.

The lack of absorption across the skin likewise was surprisisg. It was expected this would be appreciable in view of the findings of Popovici and co-

*The "initial" concentration (obtained by extrapolation back to zero time on the curves) were 0.0021, 0.00215 and 0.00245 per cent of dose, indicating that the material must have been diluted with large volumes, namely 47.6, 46.5 and 40.8 liters, respectively, in order to give such concentrations.

workers[9] which indicated there was a fall in serum calcium levels after application of the drug to the skin.

SUMMARY

This study in human beings using C^{14} labeled material indicates that Ca EDTA passes through the body unchanged. It is excreted via the kidney by both glomerular filtration and tubular excretion. The turnover time from the blood is approximately one hour after intravenous administration and one and a half hours after intramuscular injection. It quickly mixes with almost all of the body water except that it does not pass into the red cells and passes relatively slowly into the spinal fluid compartment. It is poorly absorbed from the gastrointestinal tract (a maximum of 5 per cent) and practically not at all through the skin. The use in therapeutic doses did not bring about any unusual cardiovascular changes in the cases studied.

We wish to express thanks to Ernest Anderson, Samuel Rothermel, and Thomas Moore for their aid in this work.

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THE SERUM IRON IN EXPERIMENTAL HEPATOCELLULAR NECROSIS

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IN PATIENTS with infectious hepatitis significant elevations of the serum iron have been reported,[1-7] but no evidence has been offered as to the underlying mechanism and no satisfactory histologic serial observations are available to correlate the changes in serum iron with the histopathology of the liver. The hypersideremia in infectious hepatitis is the more remarkable as it has been demonstrated repeatedly that bacterial and viral infections as well as sterile inflammation result in a marked lowering of the serum iron.

In our clinical studies[8] it was noted that the hypersideremia was present during the acute phase of hepatocellular necrosis in patients with zonal or massive hepatitis while normal serum iron levels were found during the early stages of obstructive jaundice and in patients with chronic liver cirrhosis although in many of the latter cases the hepatic function profile indicated a marked reduction in liver function. The elevation of serum iron in liver diseases thus seemed to be related to an acute disintegration of parenchymal tissue rather than to chronic parenchymal failure, and consequently should be reproducible in experimentally induced hepatocellular necrosis.

Acute parenchymal necrosis was, therefore, produced in dogs by administration of carbon tetrachloride. The animals were sacrificed at various intervals after CCl_4 had been given and the histologic findings were compared with the observed serum iron changes. The time relationship between changes in the serum iron and histologic liver changes, especially the appearance of acute hepatocellular necrosis, could thus be studied. In addition, the extent of hepatocellular necrosis was estimated from sections of various parts of the liver, and thus the quantitative relationship between the extent of acute necrosis and the absolute and relative increases in serum iron could be evaluated.

METHODS

Carbon tetrachloride was administered to 24 mongrel dogs kept on a regular laboratory diet of Nutrena dog chow supplemented by 100 Gm. of horse meat daily. Six ml. of CCl_4 per kilogram body weight was given by stomach tube in three divided doses with two-hour intervals. The animals were sacrificed from twelve hours to fifty-six days after the last dose of CCl_4. In order to avoid the diurnal serum iron variations,[9] the blood was withdrawn, as a rule, between 8:00 and 10:00 A.M. Hemolysis was prevented by puncturing

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the jugular vein with an 18 gauge needle and by collecting the blood directly in a tube without using a syringe. Only serum samples free of visible hemolysis were used for the serum iron determination which was carried out according to Barkan's method.[10] Two milliliters of serum were mixed with 1 ml. of 0.3 N hydrochloric acid and allowed to stand at 37° C. for twenty-four hours. One milliliter of 20 per cent trichloracetic acid was added and the tube was shaken vigorously. After standing for one hour, the tube was centrifuged and to 2 ml. of the clear supernatant fluid 0.25 ml. of saturated sodium acetate, 0.15 ml. of a 1 per cent freshly prepared ascorbic acid solution and 0.5 ml. of 0.1 per cent 0-phenanthroline solution were added. One hour was allowed for the development of the color and the density was measured in a Beckman spectrophotometer at 5,150 A. U. The prothrombin concentration was estimated by the one-step method and Quick's[11] concentration-time curve for dog blood was used. For the bromsulphalein test, 4 mg. of bromsulphalein was injected per kilogram body weight and the bromsulphalein serum concentration was measured on fifteen-minute specimens. A bromsulphalein concentration of 6.6 mg. per cent was considered as 100 per cent retention.

The bilirubin concentration in the serum was determined according to Malloy and Evelyn,[12] thymol turbidity according to Maclagan,[13] total protein after Phillips,[14] and the globulin fraction according to Looney.[15]

An autopsy was performed on each dog and several specimens of liver tissue from each lobe were fixed in (a) 4 per cent formalin, (b) Zenker's fluid, and (c) in absolute alcohol. Tissues were stained by Hematoxylin-eosin, Best's carmine, scarlet red, Perle's iron stain, and Schiff's periodic-acid methods. The extent of necrosis in percentage of the total parenchymal liver tissue was determined from various parts of each liver, there being an average of twelve slides per case. The Chalkley method[16] was used in the estimation of the necrotic areas; this estimation was carried out without knowledge of the experimental data by counting directly necrotic or viable liver cells which fell upon four points marked on the eyepiece in each random setting of the mechanical stage. The ratio of the number of necrotic to viable cells usually became stable before 800 cells had been counted in this fashion; according to Chalkley's work this value provides a reliable volumetric ratio between viable and necrotic tissue.

RESULTS

The serum iron values in 24 dogs before and after CCl_4 administration are presented in Table I. Beginning eighteen hours after CCl_4 had been given, significant rises in serum iron were found in all of the dogs. The serum iron usually reached its maximum during the second or third day and then gradually returned to base-line values by the end of the first week. The base-line serum iron values showed a considerable individual variation ranging from 10 to 108 micrograms, but in one and the same dog varied by less than 25 micrograms at repeated base-line determinations. The serum iron was generally lower in female dogs; the lowest base-line values were found in two female dogs with sarcoptic mange which had been included deliberately in this series.

The histopathology of CCl_4 poisoning has been described extensively by Lamson and associates;[17] as our anatomical findings were similar, only the pertinent histologic data will be presented. Fig. 1 shows two representative microphotographs, and the findings in each dog are summarized in Table II. A close time relationship will be noted between the onset of acute hepatocellular necrosis and the rise in serum iron, both beginning about eighteen hours after the oral administration of CCl_4. The maximum disintegration of

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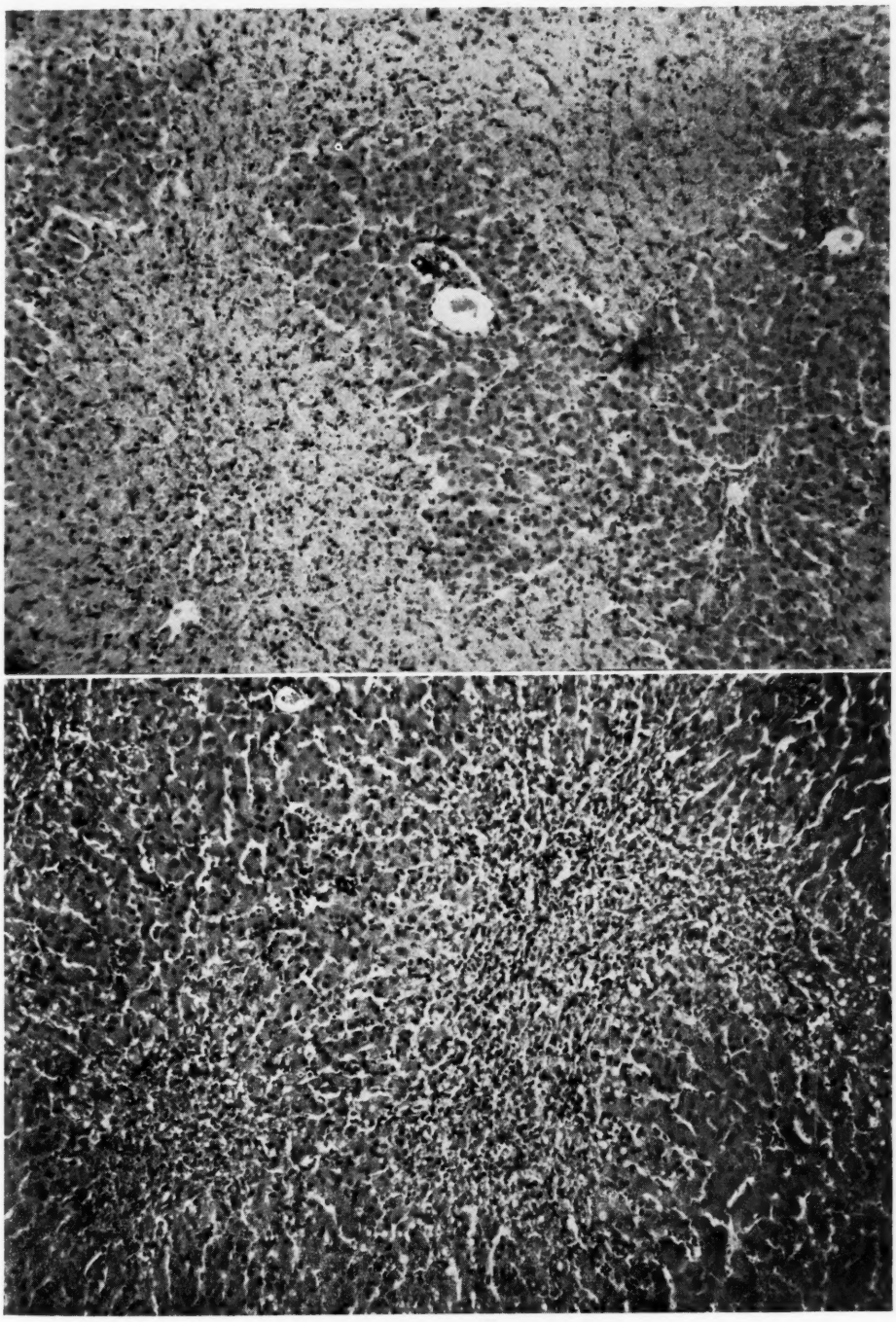
TABLE I. SERUM IRON IN DOGS BEFORE AND AFTER CCl_4 ADMINISTRATION (6 ML. PER KILOGRAM BODY WEIGHT)

DOG NO.	SERUM IRON BEFORE CCl_4 GAMMA PER CENT	SERUM IRON (GAMMA %) AFTER ADMINISTRATION OF 6 ML./KG. CCl_4																
		HOURS							DAYS									
		12	18	24	36	48	60	72	4	5	6	7	8	9	10	11	12	50
1	90, 88, 107	109																
2	16, 33, 16, 20		75															
3	66, 50, 50	120	138															
4	30, 35, 50, 35			167														
5	48, 34, 29	15	52	186														
6	52, 40, 38	58	93	93	151													
7	50, 42, 73		117		465													
8	72, 58, 83, 79			91		113												
9	23, 55, 46		79		110	355												
10	14, 10, 16, 15	82		128	73		51											
11	37, 30, 33		100		347		290											
12	112, 104, 102, 107			214		308		242										
13	28, 32, 17			18		74		112										
14	41, 27, 40		50			342		182	121									
15	52, 24, 34					47	64		48	36								
16	35, 32, 36, 34		36		67		86		141	125								
17	42, 48, 34				214		126		90		54							
18	46, 54, 65		118		154		130			112	107							
19	27, 27, 24		50		148			100	96		90	98						
20	78, 64, 64		170		260		190		130	98	93	71	74					
21	74, 91, 92	108			328		384		350			147	90	81				
22	98, 88, 82, 108			232		248		191	188			154			101		97	
23	106, 81, 102		284		294		315		290	200		111	140		120		100	83
24	84, 106, 102	118		163		196		150	130		103				96		98	89

The last serum iron listed indicates the time the dog was sacrificed.

A.



B.

Fig. 1.—*A*, Liver section in dog 7, thirty-six hours after CCl_4. The hepatocellular necrosis was estimated as 65 per cent of total tissue. The serum iron rose from 55 to 465 microgram per cent.

B, Liver section in dog No. 16, five days after CCl_4. The hepatocellular necrosis was estimated as 25 per cent of total tissue. The serum iron rose from 36 to 141 microgram per cent. Note the extensive inflammatory infiltrate which is commonly found at this stage.

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TABLE II. HISTOLOGIC LIVER FINDINGS AND SERUM IRON VALUES AFTER CCl_4 ADMINISTRATION (3 ML. PER KILOGRAM BODY WEIGHT)

DOG NO. AND SEX	1 MALE	2 FEMALE	3 MALE	4 MALE	5 FEMALE	6 FEMALE	7 FEMALE	8 MALE	9 FEMALE	10 FEMALE	11 FEMALE	12 MALE
Time sacrificed after CCl_4 administration	12 hr.	18 hr.	18 hr.	24 hr.	24 hr.	36 hr.	36 hr.	48 hr.	48 hr.	60 hr.	60 hr.	72 hr.
Average serum iron before CCl_4, microgram per cent	95	21	55	37	37	45	55	77	41	14	33	109
Highest serum iron after CCl_4, microgram per cent	109	75	138	167	186	151	465	113	355	128	347	308
Serum iron at time of sacrificing, microgram per cent	109	75	138	167	186	151	465	113	355	51	290	242
Parenchymal necrosis: Total, % of parenchyma	0*	45	30	75	70	50	65	40***	60	60	50	50
Parenchymal necrosis: Recent, 0 → ++++	0*	++**	++**	++	++	++	++	+	+	±	±	0
Polymorph. infiltration (0 → ++++)	±	++	+	+++	+	+	++	+++	++	++	++	+
Lymphocytic infiltration (0 → ++++)	0	0	0	+	±	±	+	++	+	+	+	++
Removal of debris (0 → complete)	0	0	0	0	0	+	0	+	+	Partial	Partial	Partial
Fibrous proliferation (0 → ++++)	0	±	0	++	0	0	+	++	+	+	++	++
Mitotic activity (0 → ++++)¶	0	0	0	0	0	0	±	+	++++	++	+++	++
Regeneration (0 → ++++)#	0	0	0	0	0	0	0	0	+	++	++	++

DOG NO. AND SEX	13 FEMALE	14 MALE	15 MALE	16 FEMALE	17 FEMALE	18 MALE	19 FEMALE	20 MALE	21 MALE	22 MALE	23 MALE	24 MALE
Time sacrificed after CCl_4 administration	72 hr.	4 days	5 days	5 days	6 days	6 days	7 days	8 days	9 days	12 days	50 days	56 days
Average serum iron before CCl_4, microgram per cent	24	36	37	34	41	55	26	69	86	97	86	99
Highest serum iron after CCl_4, microgram per cent	112	342	64	141	214	154	148	260	384	248	315	196
Serum iron at time of sacrificing, microgram per cent	112	121	36	125	54	107	98	74	81	97	83	85
Parenchymal necrosis: Total, % of parenchyma	30	60	20	25	40	30	35	50	70	?†	?§	?§
Parenchymal necrosis: Recent, 0 → ++++	0	0	0	0	0	0	0	0	0	0	0	0
Polymorph. infiltration (0 → ++++)	++	+	++	0	0	0	0	0	±	0	0	±
Lymphocytic infiltration (0 → ++++)	+++	+++	++	+	++	+++	+	+	+	+	±	+
Removal of debris (0 → complete)	Partial	Mostly removed	Mostly removed	Almost complete	Almost complete	Almost complete	Almost complete	Complete	Complete	Complete	Complete	Complete
Fibrous proliferation (0 → ++++)	+	+++	++	+++	++	++	+‡	+	+	+	+	±
Mitotic activity (0 → ++++)§	+	+	0	0	0	+	0	0	0	0	0	0
Regeneration (0 → ++++)‖	++	++	++	++	++	+++	+++	+++	+++	+++	++++	++++

*Extensive degeneration but no nuclear loss.
**Fatty change prominent.
***Necrosis at this time becomes well demarcated.
†Shrinkage of central zone makes evaluation difficult.
‡Fibrous tissue contains many capillaries.
§+ One mitotic figure per 4 h.p. fields; ++++ Twelve mitotic figures per 4 h.p. fields.
‖This is a gross estimation of the replacement of the necrosis zones by parenchymal cells.

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liver cells coincided with the maximum serum iron values (second or third day), and the latter began to decline when the hepatocellular disintegration had ceased and when the removal of the debris was in progress (third and fourth day). At the seventh and eighth day the removal of debris was usually complete and the serum iron had returned to base-line levels, although at that time the regeneration of liver tissue was not at all complete.

The response of the animals to the uniform dose of 6 ml. CCl_4 per kilogram body weight was variable in terms of percentage of hepatocellular necrosis which was found to range from 20 to 75 per cent of the parenchymal tissue. This is partly due to varying amounts of CCl_4 absorbed (diarrhea), but a variable individual susceptibility to CCl_4 has been noted by Lamson[17] even after parenteral administration. In our experiments this variable sensitivity was desirable because we were not concerned with the dose-response relationship but rather wanted to study how given degrees of hepatocellular necrosis are reflected in the serum iron level. In Fig. 2 the observed maximal iron values are plotted against the extent of necrosis found at autopsy. Only dogs 6 through 21 were included because the first five dogs were sacrificed at twelve, eighteen, and twenty-four hours, respectively, after the oral administration of CCl_4, i.e., before the maximum serum iron increase occurred. Dogs 22 through 24 were sacrificed so late that an evaluation of the percentage of necrosis was not feasible on account of the proliferation of parenchymal and fibrous tissue.

In the remaining 16 dogs a surprisingly good correlation was found between the maximum serum iron increase and the extent of acute hepatocellular necrosis, as seen in Fig. 2. This trend becomes even more pronounced if one considers that the two most deviating points in Fig. 2 were due to dog 6 which was sacrificed after thirty-six hours, i.e., before the maximum serum iron response probably developed, and to dog 10. The latter animal was in poor general condition and suffered from sarcoptic mange. The base-line serum iron values in this animal were very low (14, 10, 16, 15 micrograms per cent) and in spite of 60 per cent of hepatocellular necrosis, the serum iron rose only to 128 micrograms per cent.

A lack of correlation between the serum iron changes and other liver function tests has been pointed out by Peterson[6] in cases of infectious hepatitis and the same observation was made in our experiments. In Fig. 3 a representative example is given. (The data on total and fractional proteins were omitted because no significant changes were observed in these acute experiments.) The serum iron changes in the carbon tetrachloride poisoned dog occurred more rapidly than the changes in the other liver function test, i.e., the serum iron rose earlier and returned to normal values when most of the other function tests were still abnormal. In human infectious hepatitis, Peterson[6] found a bilirubinemia to be present before the serum iron increase was noted. This discrepancy may be due to the initial stage of hepatic edema in infectious hepatitis, while in CCl_4 poisoning the hepatocellular necrosis occurs without any preceding stage of hepatic edema of significant duration.

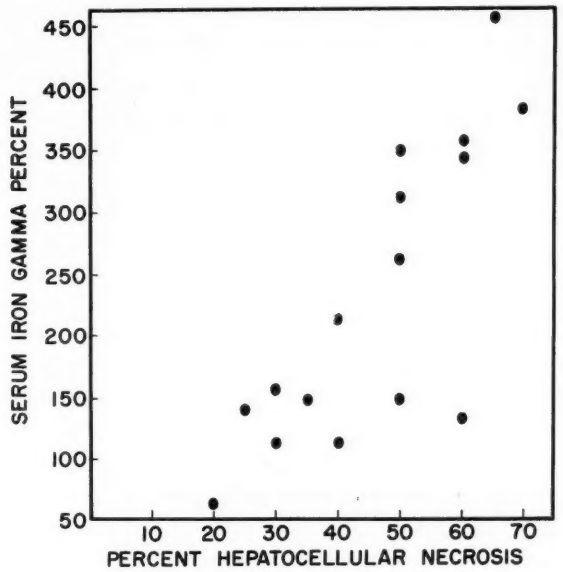

Fig. 2.—Serum iron (in microgram per cent) in 16 dogs with CCl_4 induced hepatocellular necrosis of from 20 to 70 per cent of total liver tissue.

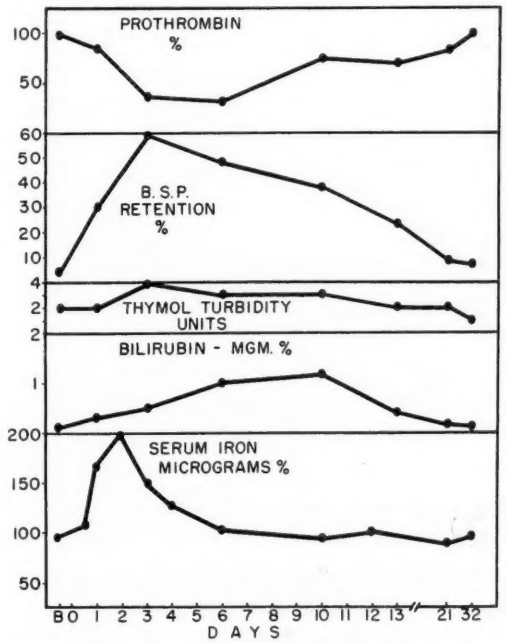

Fig. 3.—Liver function tests and serum iron in CCl_4 induced centrilobular necrosis (dog 24).

DISCUSSION

The striking time relationship between the first appearance of hepatocellular necrosis and the rise in serum iron, and between the return of the serum iron to base-line values and the cessation of parenchymal necrosis and removal of the debris, suggests that the hypersideremia is related to an actual disintegration of liver cells. In all but one animal the serum iron was found after CCl_4 poisoning to be above the control range for each animal, and in the one exception the observed necrosis amounted to only 20 per cent of liver tissue. The serum iron determination thus seems to be a reliable qualitative test in the detection of acute hepatocellular damage.

Although in most of the animals a definite trend was found to the effect that a more widespread hepatocellular necrosis was accompanied by higher serum iron levels, the scattering of the results in Fig. 2 indicates that in a given patient the absolute serum iron level does not invariably supply quantitative information on the extent of hepatocellular necrosis. The example of dog 10 suggests that among the mitigating factors to be considered are the predisease serum iron level plus concurrent infections and inflammations which tend to lower the serum iron.

The rapidity with which the serum iron follows the anatomical changes, i.e., the disintegration of liver parenchymal cells, suggests that daily or repeated serum iron determinations in patients with acute hepatitis should provide valuable information on the progress or regress of the necrotic process.

Several theories have been proposed as to the underlying mechanism. Vannotti[4] related the hypersideremia to a disturbed iron storage function of the liver, to the effect that either the iron released in the normal hemoglobin breakdown or the iron absorbed from the food cannot be stored in the liver. This explanation is difficult to reconcile with the normal disappearance rate of intravenously injected iron which was found to be identical in patients with hepatitis and in normal controls.[2, 7] In Fig. 4 an intravenous iron tolerance test is presented in a dog before CCl_4 and at the peak of hepatocellular damage; the disappearance rate of the intravenously injected iron was very similar.

Hemmler[1] suggested that the decreased excretion of iron through the bile might be responsible for the hypersideremia. In patients with obstructive jaundice, however, the serum iron levels are usually normal[8] and no serum iron increases were observed in dogs whose common ducts had been ligated.[18]

Elevated serum iron levels are known to occur in conditions with increased breakdown of hemoglobin, and it is theoretically conceivable that hemolysis takes place in the stagnant blood in the sinusoids of the necrotic liver areas. A considerable degree of hemolysis, however, would be necessary to explain the observed serum iron increases, and a hemolysis of such magnitude should be reflected in changes in the hemoglobin concentration and reticulocyte count; no such changes were observed in human infectious hepatitis or in these experiments.

The close time relationship found between the actual disintegration of liver cells and the peak of hypersideremia supports as a possible explanation a release of iron from the dying liver cells. The disappearance rate of excess iron from the blood stream as obtained in the iron tolerance test (Fig. 4) permits a gross estimation of the order of magnitude of the amount of iron that must be released from the dying liver cells in order to produce the observed hypersideremia. Approximately 150 gamma of iron disappeared during the first two hours per 100 ml. of plasma, or 75 gamma per hour. Assuming a plasma volume of 500 ml. in this dog, 5 times 24 times 75 gamma or a total of 9 mg. of iron per twenty-four hours would theoretically be necessary to compensate for the disappearance and to maintain the level of 350 microgram per cent. The disappearance rate of iron gradually released from dying liver cells may differ considerably from the intravenously injected iron, but this gross estimation indicates that the required breakdown of liver tissue would be compatible with the actually observed extent of necrosis, because 100 Gm. of liver tissue contain between 10 and 30 mg. of iron.

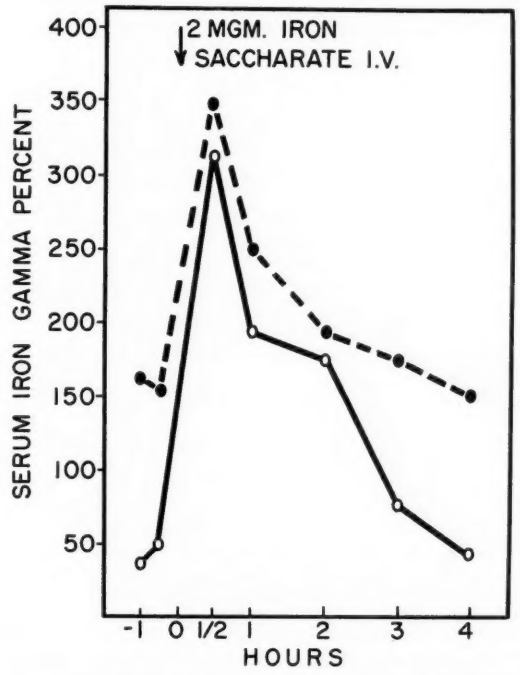

Fig. 4.—Iron tolerance test (2 mg. iron intravenously) in a dog before (○——○) and after (●— — —●) CCl_4 induced hepatocellular necrosis.

SUMMARY

Hepatocellular necrosis was produced in 24 dogs by oral administration of 6 ml. CCl_4 per kilogram body weight. In all animals significant increases in serum iron were observed and there was a close time relationship between the rise in serum iron and the appearance of hepatocellular necrosis. The

serum iron rise usually began eighteen hours after CCl_4 administration and reached its maximum on the second or third day. The iron values decreased during the fourth and fifth day parallel with the cessation of the disintegration of liver cells and returned to base-line values at the end of the first week when the removal of the cellular debris was found to be almost complete.

The extent of hepatocellular necrosis varied between 20 and 75 per cent of liver tissue, and in most animals a good correlation was found between the maximum serum iron level and the extent of necrosis in percentage of total hepatic tissue. The average serum iron increases in percentage of base-line values were found to be 173 per cent in one animal with 20 per cent necrosis, 353 per cent in animals with 25 to 30 per cent necrosis, 469 per cent in the group with 35 to 50 per cent necrosis, and 629 per cent in the group with 60 to 75 per cent hepatocellular necrosis.

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EFFECTS OF AMINOPTERIN ADMINISTRATION ON THE RESPIRATION AND MORPHOLOGY OF THE GASTROINTESTINAL MUCOSA OF RATS

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THE intestinal mucous membrane is one of the more active metabolic tissues of the body. Although various nutritional deficiencies produce gastrointestinal lesions, few studies have been made of the effects of these deficiencies on the metabolism or on the pathogenesis of benign or malignant lesions of the gastrointestinal mucosa. A recent paper from this laboratory[1] has demonstrated the relationship between intestinal bleeding and lowered respiratory rates of the mucosa in pantothenic acid deficiency. The necessity for appropriate dietary control to demonstrate the specific effects of pantothenic acid deficiency was also shown.

Much clinical interest in folic acid antagonists was simulated by the work of Farber and associates[2] on the treatment of leukemia in children with Aminopterin (4-amino-pteroyl-[L]+glutamic acid). These studies and those of others revealed the gastrointestinal effects of this antivitamin, including nausea, vomiting, melena, and diarrhea in human beings,[2, 3] and destruction of the epithelial elements in experimental animals.[5] Preliminary studies on its use in gastrointestinal cancer have been made.[6]

The present study is concerned with changes in the metabolism of duodenal mucosa obtained from rats treated with Aminopterin and the concomitant morphologic changes. The need of morphologic studies for the interpretation of certain metabolic changes was made clear in these and other studies.[1]

EXPERIMENTAL

Adult female rats from the Charles River Breeding Laboratories were used in all experiments. The various groups of animals were fed one of the diets listed in Table I. It has been demonstrated[1] that in order to show the specific effect of pantothenic acid deficiency on duodenal mucosa respiration, diets high in protein have to be fed to the experimental animals. For this reason, high-protein diets were included in these studies (Table I). The animals were injected subcutaneously with Aminopterin dissolved in saline. They were sacrificed by decapitation, and 10 to 12 cm. of duodenum were removed, cooled on ice, opened lengthwise, and washed with saline. The mucosal tips were

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scraped off with a Stadie blade. Little crypt epithelium was included, as confirmed by repeated histologic study. The remaining portion of the tract was also opened and examined for gross lesions and promptly fixed in formalin. The formalin-fixed tissues were rolled into a spool for histologic examination. The spools were embedded in paraffin according to the picric acid-dioxane method [7] cut in a routine manner, and stained according to Mallory's method with aqueous alum hematoxylin and phloxine B. Oxygen uptake by the villus tips was measured, using the Warburg technique. Approximately 60 to 100 mg. of tissue were used per flask. Glucose, acetate, or pyruvate in concentrations of 36.0 mM per liter were added as substrates in a modified Krebs phosphate media.[8] Carbon dioxide was absorbed by potassium hydroxide in the center well and the gas phase was pure oxygen. After an equilibrium period of five minutes, oxygen uptake was measured at 37° C. for one hour. At the end of the run, the tissue was filtered off on a tared piece of filter paper, dried at 110° C., and weighed to obtain the weight of the dry tissue. The results on oxygen uptake are expressed as QO_2 (μl of oxygen consumed per milligram of dry tissue per hour).

TABLE I. COMPOSITION OF RATIONS

CONSTITUENTS	BASAL	HIGH PROTEIN
	Gm. per 100 Gm. of Diet	
Casein (vitamin free)	18	60
Salt mixture IV (9)	4	5
Cod liver oil (U.S.P.)	1	1
Lard	4	4
Choline chloride	0.3	0.3
Sucrose	72.7	29.7
	Mg. per 100 Gm. of Diet	
Nicotinic acid	4	4
Riboflavin	0.8	0.8
Pyridoxine hydrochloride	0.4	0.4
Thiamine hydrochloride	0.4	0.4
Calcium pantothenate	2	2

TABLE II. EFFECT OF AMINOPTERIN ON SURVIVAL TIME OF RATS AND RESPIRATION OF DUODENAL MUCOSA FROM TERMINAL ANIMALS

AMINOPTERIN DOSE (μG/KG. BODY WEIGHT)	NO. ANIMALS	DIET FED	SURVIVAL TIME (DAYS)	QO_2 μL O_2/MG./HR.
		Basal		
0	3	High Protein	—	14.2+
240	10	Basal	4-5	
240	10	High protein	6-8	14.8 + 1.8
120	10	Basal	8	
120	10	High protein	10-12	—
60	10	High protein	17	
60	10	High protein + .3 per cent cystine*	23	—

*Replacing an equivalent amount of sucrose.

RESULTS

The results of the first series of studies are shown in Table II. Animals given a daily dose of 240 μg of Aminopterin per kilogram were sacrificed at the end of approximately six days when they were about to die. The metabolism of duodenal mucosa from these animals was normal (QO_2 of 14.8). That this normal rate of respiration failed to reflect marked tissue damage was revealed when microscopic sections of the mucosa were subsequently re-

viewed, as described later. The survival time of the animals given Aminopterin was approximately proportional to the dose. Animals given daily doses of 120 μg per kilogram survived approximately nine days. Half of this dose increased the survival time about twofold. The feeding of high-protein diets to animals afforded little protection against the toxicity of Aminopterin administration. The addition of cystine to the high-protein diets, however, may have offered some protection (Table II).

Regardless of the diet fed and the dose of Aminopterin given, all animals, when terminal, exhibited watery diarrhea, rough coat and, in most cases, a gastrointestinal tract filled with blood. These signs first appeared four to five days before death. Since histologic studies revealed complete epithelial destruction in terminal animals, others were sacrificed at intervals after Aminopterin injection. Two groups of rats were given daily injections of Aminopterin of 120 and 240 μg per kilogram of body weight, and three rats from each group were sacrificed daily. All animals were fed the basal diet ad libitum. Figs. 1 and 2 illustrate the results of these experiments.

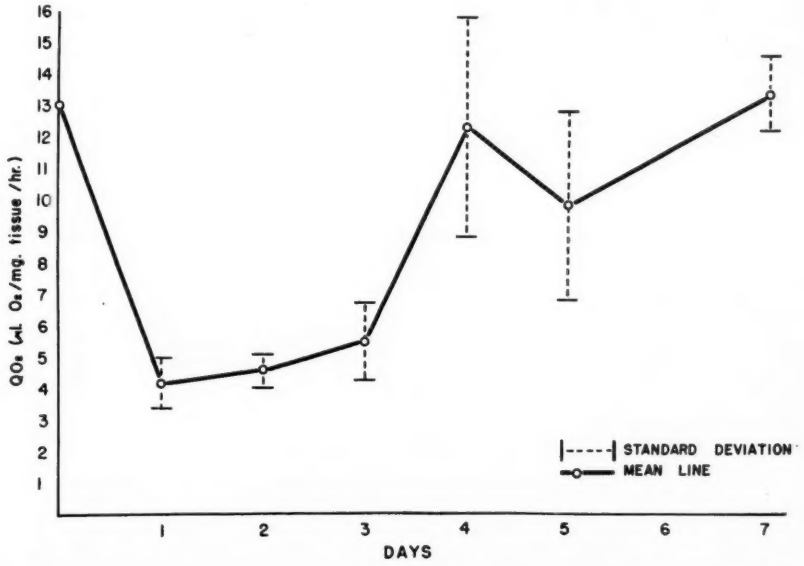

Fig. 1.—Effect of Aminopterin on duodenal mucosa respiration (dose = 240 μg per kilogram of body weight per day). Each point represents mean of three animals.

The tissue from control animals had an average QO_2 of 12.3 with a standard deviation of 1.2. Twenty-four hours after the initial administration of 240 μg of Aminopterin per kilogram (Fig. 1), the respiration rate of duodenal mucosa fell approximately 65 per cent. The respiration of duodenal mucosal tissue from the group given 120 μg per kilogram daily (Fig. 2) was reduced only 20 per cent in the same period of time. After forty-eight hours, however, the respiration had fallen to the same level found with the higher dose at twenty-four hours. On the third day there was no apparent difference in respiration in these two groups. Although the administration of Aminopterin

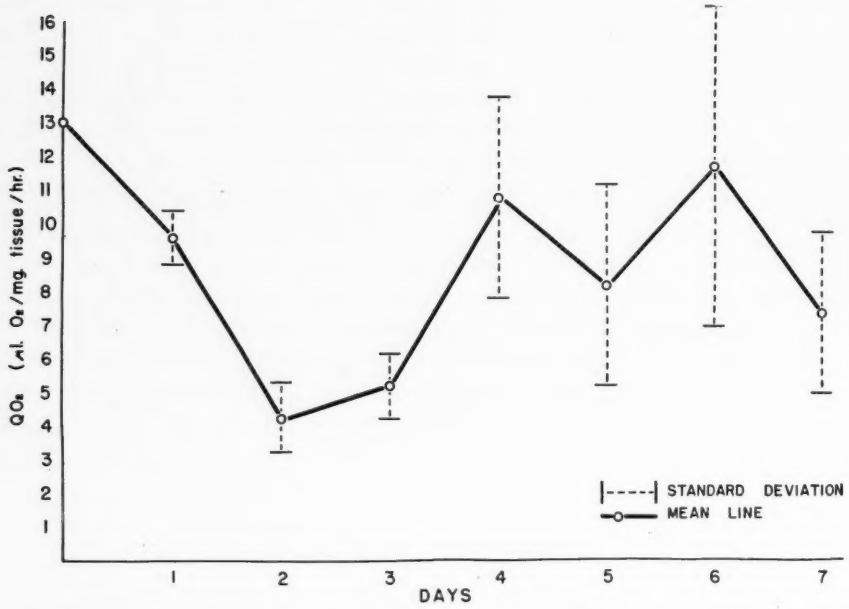

Fig. 2.—Effect of Aminopterin on duodenal mucosa respiration (dose = 120 μg per kilogram of body weight per day). Each point represents mean of three animals.

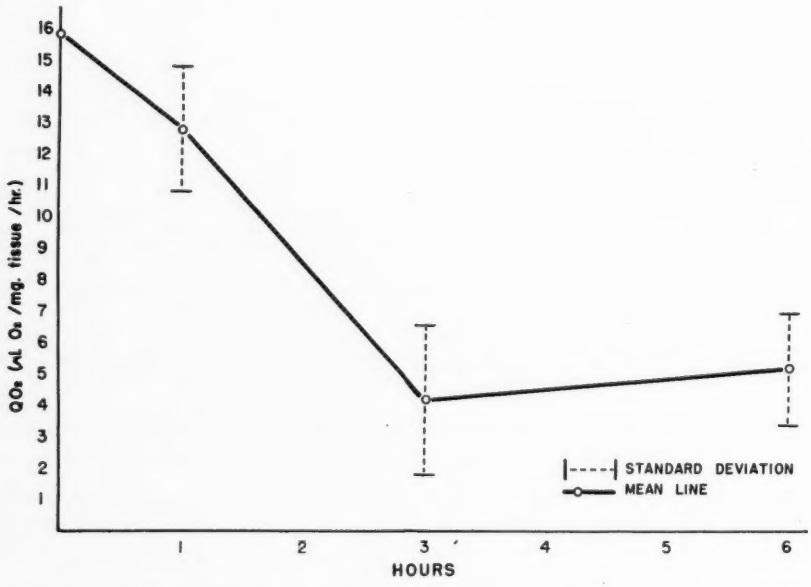

Fig. 3.—Effect of Aminopterin on duodenal mucosa respiration (single dose of 480 μg per kilogram of body weight). Each point represents mean of three animals.

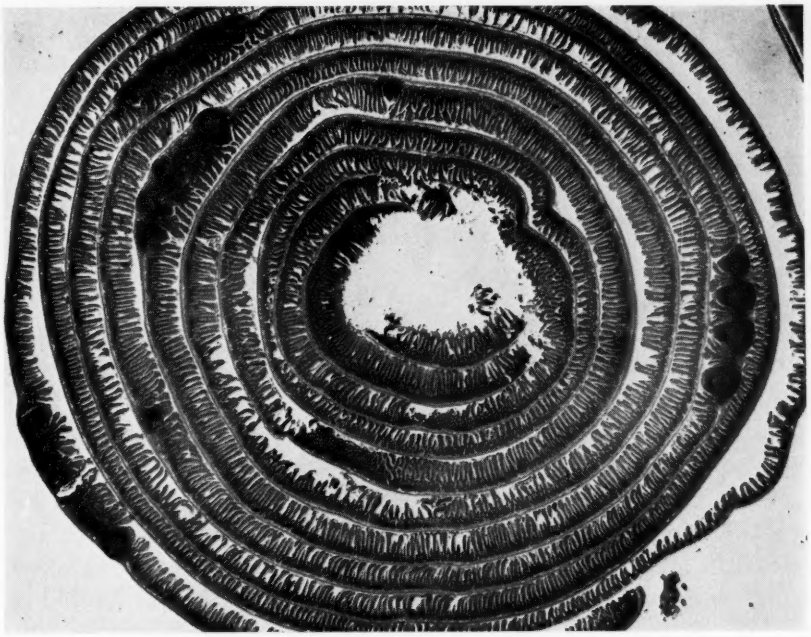

Fig. 4.—A spool of normal rat intestine showing the prominent lymph patches. (Low power.)

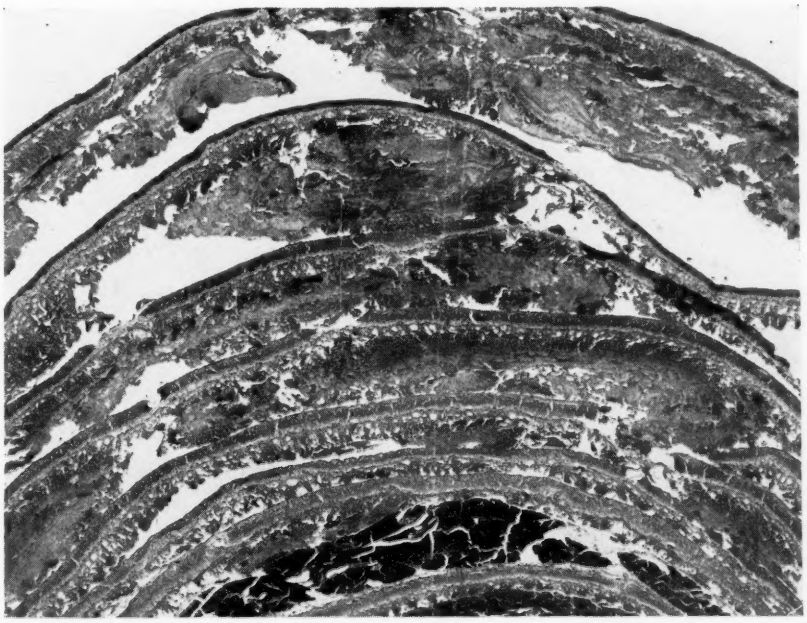

Fig. 5.—Complete loss of normal structure. Rat given 120 μg per kilogram Aminopterin daily and sacrificed when terminal on tenth day. Compare with normal intestine (Fig. 4). (×30; reduced ¼.)

was continued, the mean QO_2 values rose definitely on the fourth day. On this and succeeding days many samples showed values that were normal or even somewhat above those usually found for normal animals. It will be seen from the figures that whereas the standard deviation was small and uniform up to the third day, the variation became much larger on the following days.

Fig. 3 illustrates the effect of a single dose of Aminopterin on duodenal mucosal respiration. Animals given a single injection of 480 μg of Aminopterin per kilogram of body weight were sacrificed after one, three, six, and forty-eight hours for respiration studies. There was a slight drop in respiration after the first hour and by the third hour the mean QO_2 had fallen to the same minimal value obtained with the smaller doses after twenty-four or forty-eight hours. By the forty-eighth hour, the rate of respiration had risen to an essentially normal level, approximately 10 μl of O_2 per hour.

Pathologic Findings.—After fixation in formalin, the tissues were rolled into a spool for histologic examination (Fig. 4). Five or six such spools permitted examination of the entire small intestine. Pathologic changes were dependent on the dose of Aminopterin and the duration of survival. When a dose of 480 μg of Aminopterin per kilogram of body weight was given, the first changes occurred at approximately four and a half hours and consisted of a marked reduction in the mitotic figures normally found in the crypts of Lieberkühn of the small intestine (Fig. 6). At the end of six hours, absence of normal mitoses was noted. Instead, the crypts showed degenerated mitotic figures, nuclear remnants, and disintegrated cells (Fig. 7). The epithelium of villus tips, however, was not markedly altered. At the end of forty-eight hours the crypts again showed abundant normal mitoses. When repeated smaller doses of Aminopterin were given, the absence of mitotic activity coincided with the lowest respiration values, and, when an increase in respiration was observed, abundant mitoses were again found in the crypts. When the animal was sacrificed just before death (varying from four to twenty days depending on the daily dose), striking pathologic changes were noted. Figs. 8 and 9 show the effect of 480 μg of Aminopterin repeated daily. The tunica propria of the duodenum was heavily infiltrated with polymorphonuclear leukocytes which tended to be clustered in large numbers in the crypts. The epithelium was thin, elongated, the nuclei variable in size and shape. Mitoses in the crypts were markedly reduced in numbers or entirely absent. After the fourth day (Experiments 2 and 3) normal ribbons of epithelium were largely replaced by strands of elongated, swollen, or shrunken distorted cells (Fig. 10). These sometimes accummulated in clusters and syncytial masses rather than in normal columns. It was apparent that we were measuring the respiration not only of epithelium, normal and damaged, but also of white cells which normally respire actively and of connective tissue which is metabolically inactive.

Characteristically, in advanced lesions the crypts became swollen and distended with fluid; mitoses disappeared and the epithelium became flattened and cuboidal rather than columnar. In more advanced lesions the

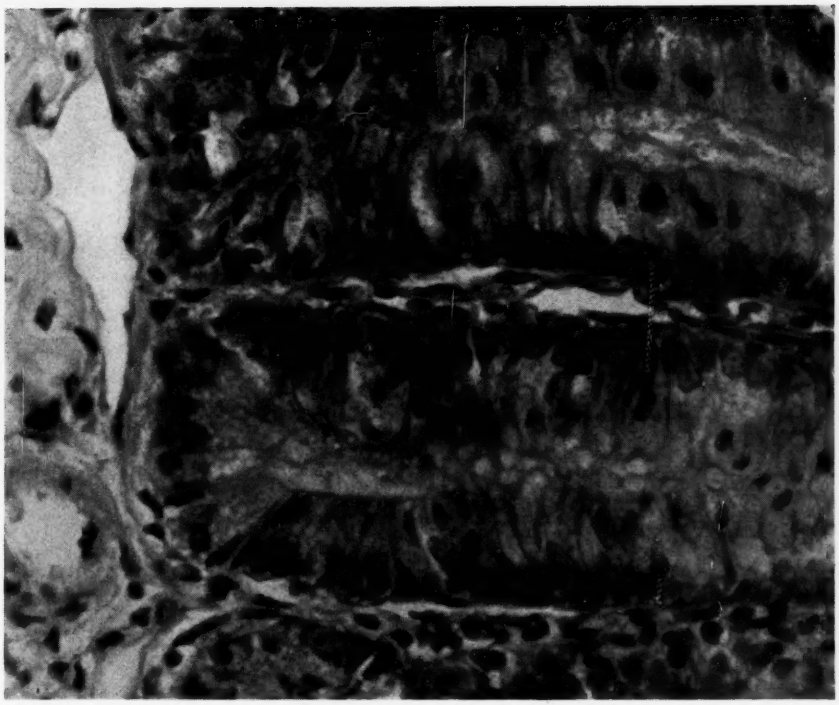

Fig. 6.—Normal intestine showing abundant mitoses in duodenal crypts. (Lumen of crypts to right.) (×800; reduced ¼.)

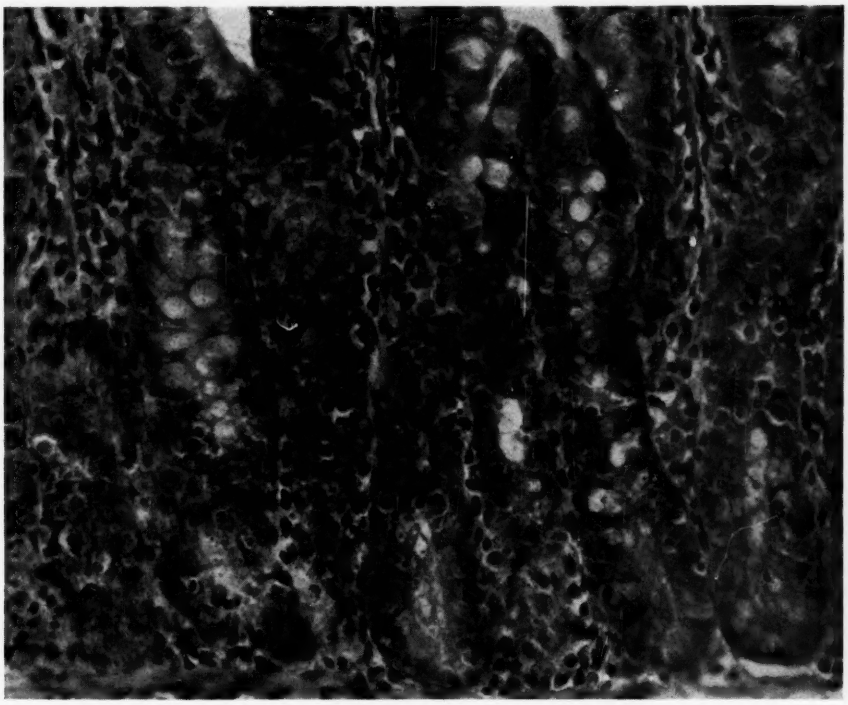

Fig. 7.—Replacement of normal mitoses by degenerated cells, nuclear remnants and debris in rat sacrificed six and one-quarter hours after injection of 480 μg per kilogram Aminopterin. (Lumen of crypts to top.) (×400; reduced ¼.)

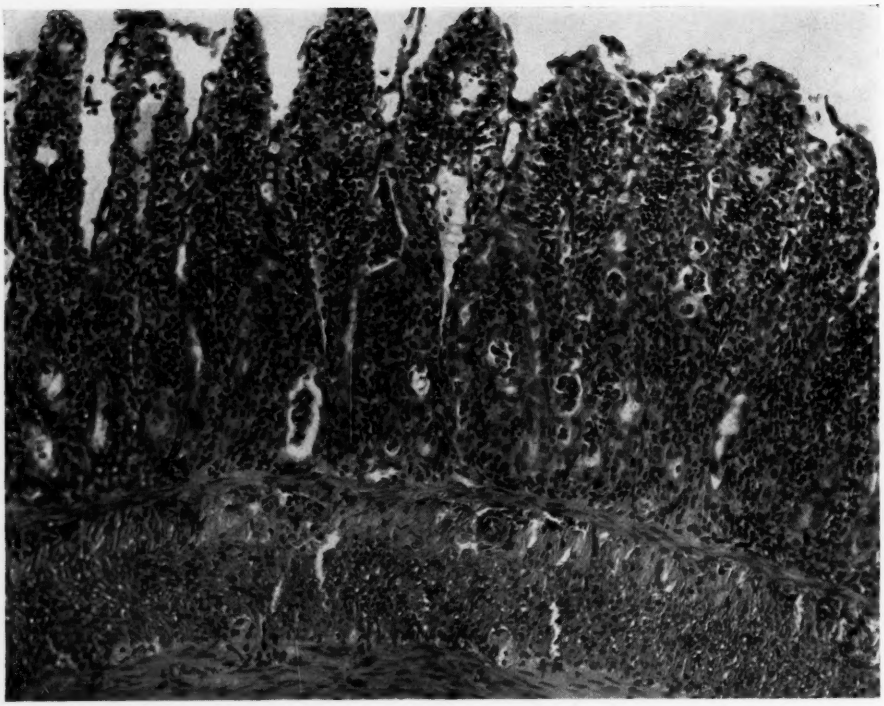

Fig. 8.—Duodenal villi heavily infiltrated with white blood cells. Crypts free of mitoses and plugged with white blood cells. Mucosa flattened. Aminopterin, 480 μg per kilogram daily for four days. Animal moribund. (×200; reduced ¼.)

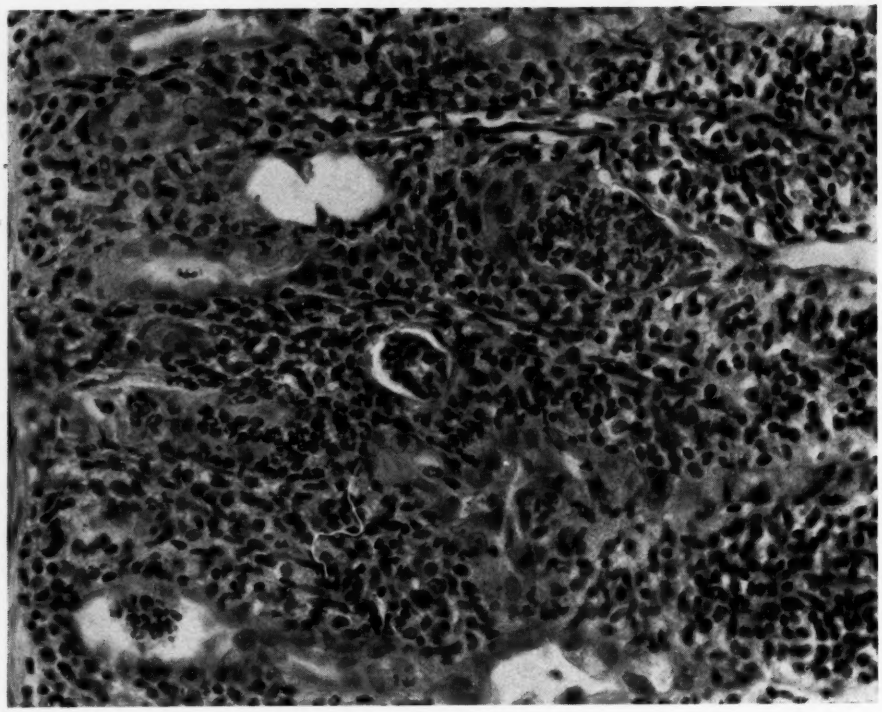

Fig. 9.—Duodenal villi heavily infiltrated with white blood cells. Crypts free of mitoses and plugged with white blood cells. Mucosa flattened. Aminopterin, 480 μg per kilogram daily for four days. Animal moribund. (Opening of crypts to right.) (×450; reduced ¼.)

epithelium both in the crypts and in the villus tips disappeared entirely (Fig. 11), and in still further advanced lesions the entire mucous membrane of the intestine was replaced with inflammatory and hemorrhagic elements (Fig. 5). Brunner's glands in the duodenum were usually not as badly damaged as was the overlying crypt and villus epithelium. Similarly, epithelium immediately overlying lymphoid patches tended to be spared. Lesions tended to occur most rapidly and extensively in the lower segments of the upper half of the small intestine, although this was not always the case.

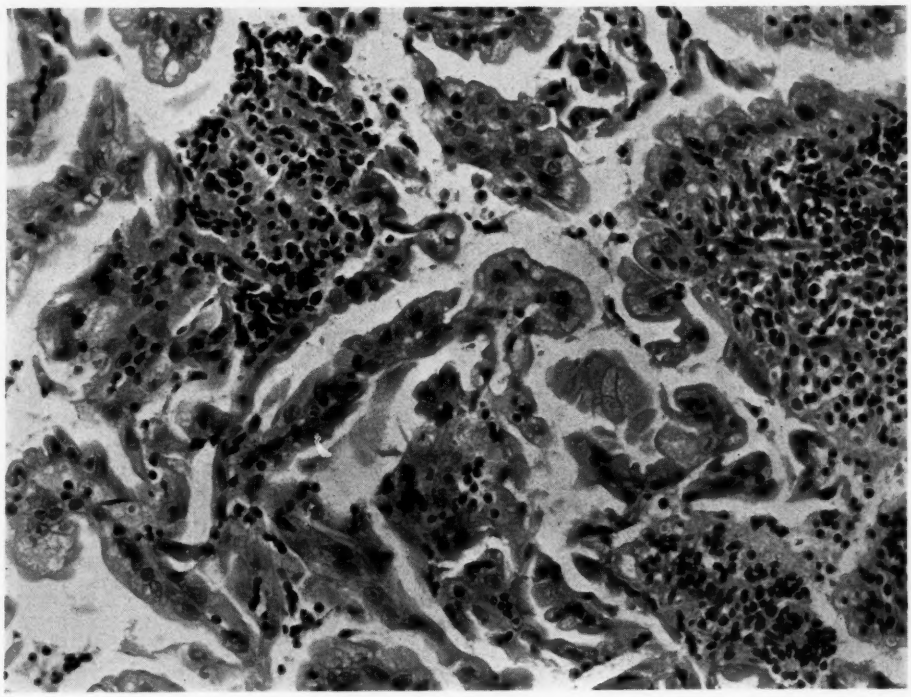

Fig. 10.—Duodenal mucosal scraping showing effect of Aminopterin. Distorted mucosal cells, dense tunica propria (connective tissue) infiltrated with round cells. Aminopterin 240 μg per kilogram daily; sacrificed fourth day. (×400; reduced ¼.)

Thus, the variation in respiration observed on the fourth and following days could be explained in part by the morphologic studies. Some tissue taken at this time was composed largely of badly damaged cells of presumably low respiratory rate. Other samples were composed of tissue of high respiratory rate such as less severely damaged tissue, regenerating tissue, or tissue infiltrated with white cells. Other factors were undoubtedly important, but it seems clear that the variability in the respiration of the tissue was in part a reflection of the variability in the composition of the tissue studied.

The injection of a single dose presented a different situation from that of repeated daily doses. Although the maximum drop in respiration was dependent upon the size of the dosage, there was no permanent injury to the tissue, either metabolically or morphologically. It was not possible to kill

an animal with a very large single dose given intraperitoneally. An injection of 1,000 μg Aminopterin per kilogram caused no apparent discomfort to animals.

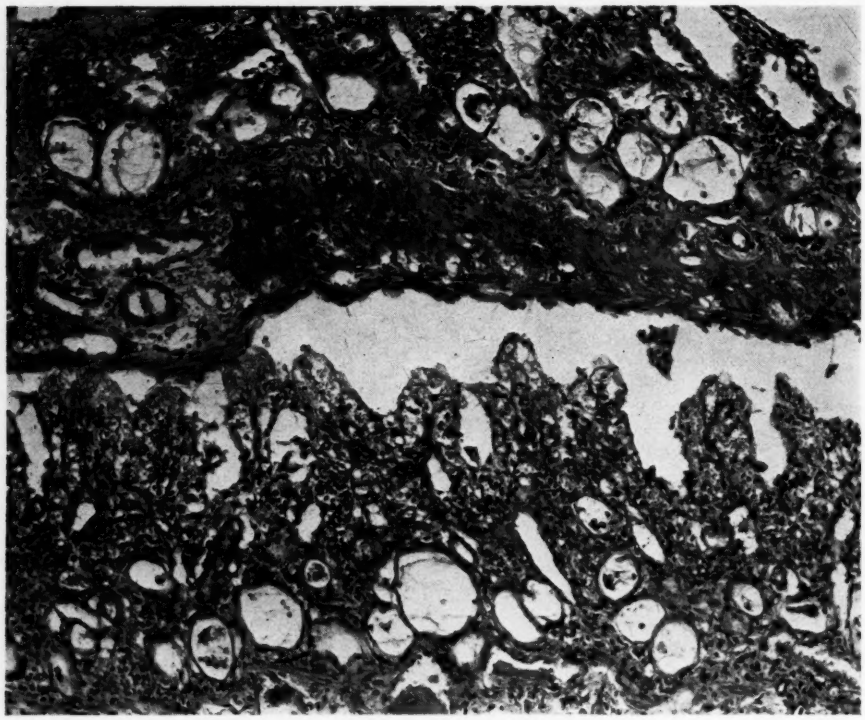

Fig. 11.—Characteristic cystic appearance of duodenum in advanced damage. No normal epithelium is seen. Crypts appear as dilated cysts filled with inflammatory cells and non-staining fluid. Aminopterin 240 μg per kilogram daily; sacrificed fourth day. (×200; reduced ¼.)

DISCUSSION

Although folic acid or the related citrovorum factor has been implicated in several enzyme systems,[10] no specific metabolic role can as yet be ascribed to these materials. Similarly, the metabolic and pathologic effects of Aminopterin described in this paper cannot be related to specific enzyme systems blocked by Aminopterin, although this is presumably the basis of its action. Maximum reductions in respiration of villus tip epithelium coincided consistently with the absence of normal mitotic activity at the villus bases. Final return of mitotic activity confirms the earlier report of Grampa and Dustin.[11] Le Blond and Stevens[12] have shown that the crypt cells, where mitoses are observed, are precursors of villus cells above. They calculated an approximate thirty-six-hour survival for a mucosal cell between the onset of mitosis in the crypt and the final extrusion of the cell into the gut lumen.

The reduced respiration obtained from villus tip epithelium probably reflects both a direct effect of Aminopterin on the villus tip mucosa and an

indirect effect due to interference with production of cells in the crypts. Although the earliest change occurred in the crypt epithelium, the site of mitotic activity, the respiration of the crypt cells has not yet been studied.

Many investigators have studied the in vitro metabolism of various tissues using various substrates without morphologic control. Without morphologic observations, however, the profound metabolic alteration induced by Aminopterin might have been overlooked since mucosa of terminal animals had essentially normal rates of oxygen uptake (Table II). It is evident that when one is dealing with a deficiency that occurs rapidly or with a tissue that has a rapid rate of turnover, or both, time becomes an important factor. Weight loss is commonly used as a criterion for the onset or the degree of a nutritional deficiency. Many of the animals used in these experiments lost relatively little weight. The rise in the rates of oxygen uptake by duodenal mucosa from animals given 120 and 240 μg per kilogram per day (Figs. 1 and 2) coincided with the onset of severe signs of diarrhea, rough coat, and melena. Had single observations been made at this time it would have been difficult to reconcile the metabolic results with the obviously critical state of the animals.

Gastrointestinal symptoms including diarrhea have been observed in nutritional deficiencies in man, including sprue and pellagra. The poor state of such patients has sometimes been attributed to the severe diarrhea. Little attention has been devoted to the effect of the deficiency on the metabolism of the intestinal mucosa itself.

SUMMARY

The QO_2 for normal duodenal mucosa is approximately 12 to 16 μl per milligram per hour. Beginning as soon as one hour after subcutaneous injection of a large dose of Aminopterin, there was a drop in the rate of oxygen uptake, the extent and duration of which depended upon the dose. Morphologic changes lagged behind the metabolic changes and were most marked after the fourth day and when animals were terminal. Pathologic changes were extensive and included the following: (1) reduction of crypt mitoses, sometimes followed by cellular necrosis and fragmentation; in later stages, swelling of crypt lumina with flattening of crypt epithelium and distention with edema and inflammatory cells; (2) infiltration of tunica propria with inflammatory cells; (3) degeneration of villus tip epithelium and, in terminal animals, complete denudation of epithelium, and hemorrhage.

When the respiration rate of villus tip epithelium was lowest, there was almost complete absence of normal mitotic activity in the crypts. After a time lag, respiration increased and mitosis returned.

On the fourth and following days a rise was seen in the villus tip respiration despite continued antifolic administration. At this time variation between individual determinations was great. This may have been due in part to variation in structure of incubated tissue caused by variable amount of degeneration, regeneration, and infiltration.

These studies emphasize the value of morphologic controls in metabolic investigation.

We wish to express our appreciation to Dr. Sidney Farber and Dr. Israel Diamond for their helpful suggestions and to Miss Lillian Leavitt, Mr. Thomas Faherty, Miss Helen McGrath, and Miss June Nay for their technical assistance. Mr. Leo Goodman prepared the photographs.

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EFFECT OF EARLY EXSANGUINOTRANSFUSION ON IRRADIATED DOGS

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INTRODUCTION

THE effect of ionizing radiation on the living organism appears to be the result of two different processes: first, a direct trauma inflicted on the cells by the primary rays or their secondary particles; second, an indirect damage caused by toxic agents, e.g., necrohormones[1] which are produced during and/or following exposure and which are carried by the body fluids from one region of the organism to another. These substances, being probably nonspecific for radiation,[2] are believed responsible for changes in tissues far removed from the direct site of radiation.[3-5] Thus, the indirect part of radiation injury might be reduced by elimination of the toxic agents from the organism in replacing the blood of irradiated animals by that of healthy donors or by suitable blood substitutes. To obtain an almost complete replacement of the irradiated animal's blood, the method of exsanguinotransfusion was utilized in the present study.

Since 1916,[6] exsanguinotransfusion has been tried in the treatment of various types of chemical intoxication. During the last decade replacement transfusion has become a standard clinical procedure in the therapeutic management of erythroblastosis fetalis, as a result of the studies of Wiener and colleagues,[7] Wallerstein,[8] Diamond,[9] and other workers. An excellent review and mathematical treatment of different exsanguinotransfusion methods has been given by Wallerstein and Brodie.[10] For the experimental conditions and requirements of the present work, a modification of Diamond's method of intermittent substitution transfusion with equal volume replacements was found to be most adaptable.

METHOD

Animals.—Forty mongrel dogs of approximately 7 kilograms were exposed to x-radiation. The animals were divided into two groups. Each group contained 20 dogs, of which 15 were male and 5 female. Following irradiation, the first group (control group) received no treatment; whereas, the second group (treated group) was exsanguinotransfused. The animals had access to Purina Dog Chow and water, ad libitum.

Irradiation.—The dog received a sedative dose of Nembutal and was immobilized in a standing position by means of a leather harness and an iron frame. The immobilization was found to be necessary to insure exposure of the animal to the exact dose of x-radiation desired. The skin-target distance was 118 cm. At this distance, the Picker x-ray unit produced a dose rate of approximately 13 r. per minute in air under the conditions of the experiment (voltage, 260 KVP; amperage, 18 Ma.; inherent filtration, 0.25 mm. copper;

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additional filtration, 0.5 mm. copper and 1.0 mm. aluminum). After exposure of the dog to this intensity for sixteen minutes, the iron frame was rotated horizontally through 180 degrees, and the animal irradiated for another sixteen minutes; thus, a relatively homogeneous distribution of radiation effects was obtained throughout the whole body of the animal. The total dose was 420 r. in air.

Blood.—Healthy, large, mongrel dogs of about 20 kilograms were used as blood donors. Approximately twenty-four hours prior to a particular radiation experiment 3 dogs were anesthetized and the right jugular vein of each was exposed under sterile conditions. Into this vessel a polyethylene tube was inserted as far as the entrance of the right atrium. The tube was then connected to a 650 ml. vacuum bottle containing 50 ml. of 4 per cent sodium citrate. With continuous agitation of the bottle, 20 ml. of blood per kilogram of body weight were withdrawn. This procedure was found to be the most satisfactory way of obtaining large amounts of blood without encountering any difficulties caused by clotting or collapsing of the vessel. The total quantity of blood withdrawn from the 3 donors amounted to roughly 1,100 ml. The blood was pooled and stored in a refrigerator at 4° C. for twenty-four hours.

Exsanguinotransfusion.—Within two hours following irradiation of a particular pair of dogs, one of the animals received into a vein of the foreleg an anesthetic amount of 3 per cent Nembutal. Then, a polyethylene tube was inserted into the jugular vein in the same way as described above for the withdrawal of blood from the donors. Under the assumption that the total blood volume of the dog amounted to one-thirteenth of the body weight, half of this amount was withdrawn into a vacuum bottle (Fig. 1). After completion of this procedure, the polyethylene tube was connected to a bottle containing the stored blood. The quantity of blood removed during the first phase was fully replaced by donor blood at body temperature by gravity drip (Fig. 2). This step of intermittent withdrawal and replacement was repeated until the donor blood received amounted to twice the estimated total blood volume of the recipient. In order to counteract the hypocalcemic effects of the citrate ions, 10 per cent calcium gluconate was administered intravenously 1 ml. per 100 ml. of donor blood infused. Finally, an excess of 50 to 100 ml. of blood was administered, the polyethylene tube removed, and the incision closed. Heparinization of the animals was not necessary.

Expressed as percentage of donor blood present in the circulatory system of the recipient at completion of the exsanguinotransfusion, the efficiency of the intermittent substitution method is described by the following equation:

$$P = 100 \left[1-(1- \frac{v}{V})^{n} \right]$$

where,

P = percentage of donor blood present in the recipient;
V = total blood volume of the recipient;
v = blood volume exchanged in each step;
n = number of steps.

From the average body weight of the dogs used, V can be estimated as approximately 500 ml. (1/13 of 7 kg.). Therefore, v is close to 250 ml. (½ total blood volume, V), and n is equal to 4. With these values, P is found to be 93.8 per cent. Hence, about 90 per cent of the recipient's blood can be expected to be replaced by donor blood.

Cross-Matching.—Many antigenic factors may be present in dog erythrocytes. Thus far, six have been studied extensively[11, 12] and are designated A, B, C, D, E, and F. In order to prevent most of the hazards of incompatibility in transfusions, it is sufficient to test red corpuscles of prospective donor and recipient dogs only for the presence or absence of the canine A factor.[13] For this present study anti-A dog serum was not available; thus, only a cross-matching at room temperature was performed between washed red cells and serum of the prospective donors and recipients. Although dogs withstand incompatible transfusions remarkably well,[12] a severe reaction may sometimes occur.

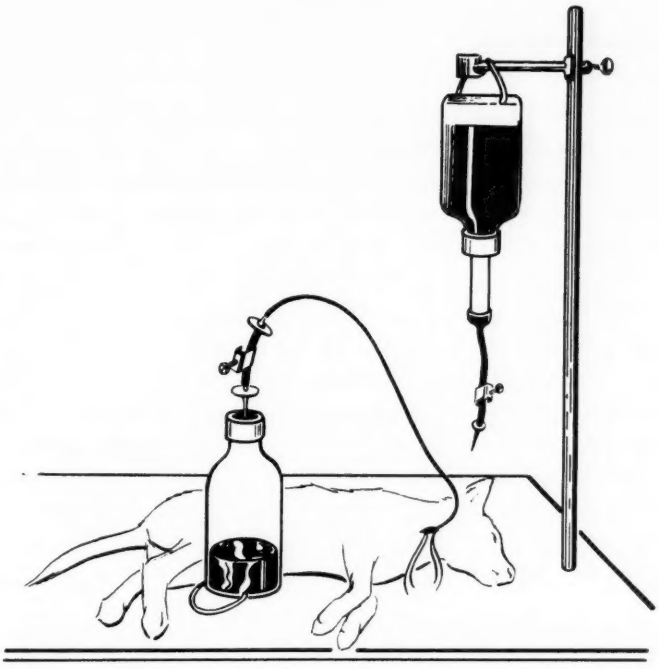

Fig. 1.—Exsanguinotransfusion of dogs. First phase of first step; withdrawal of blood.

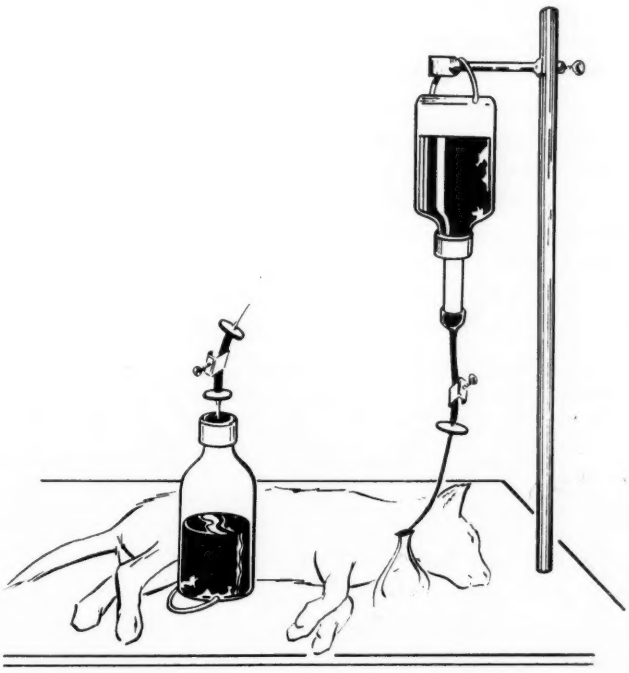

Fig. 2.—Exsanguinotransfusion of dogs. Second phase of first step; replacement of blood.

RESULTS

General Clinical Course.—

Control Group: In the development of radiation sickness three well-defined phases were observed: an initial shocklike reaction, an intermediate period of latency, and an acute, terminal stage.

Following irradiation, the animals recovered rapidly from the effect of the sedative dose of Nembutal, but within a few hours signs of nausea (with actual vomiting in half the cases), a decrease in activity, and anorexia developed. By the next day, the initial reaction vanished, and the animals displayed a relatively normal appearance, generally persisting throughout the first post-radiation week. Within this period of latency, nevertheless, an increasing listlessness and emaciation, a reduction of food and water intake, and a transitory diarrhea revealed the progressive pathologic course. After the latent period, radiation sickness rapidly entered the acute, terminal stage, which was characterized by severe prostration, total abstinence from food and water, bloody diarrhea, excessive sanguineous salivation, and ulceration of skin and buccal mucosa. Within three days following the onset of the acute phase, the animals succumbed. None of the control dogs survived the second postirradiation week.

Treated Group: Approximately one hour was required for the achievement of a 90 per cent exsanguinotransfusion. During this procedure, 3 of the 20 dogs exhibited an immediate reaction characterized by generalized muscle tremor and hyperpnea. The 3 animals died a few hours after completion of the transfusion. It was not determined whether death was caused by an inability of the cardiovascular system to adapt to the rapid changes in blood volume and/or by incompatibility of blood. However, the latter cause was suggested by the fact that 2 of these animals, as exceptions to the general procedure, received blood of a single donor; thus, the hazard of incompatibility was much greater than in those recipients whose transfusions were performed with pooled blood.[6] The remaining 17 treated dogs recovered from the anesthesia and operation within two to eight hours after completion of the transfusion. Their appearance subsequently was essentially the same as that of the corresponding controls. The surgical wound healed without any complications. No difference could be observed in the general clinical course of the treated and the control group,

TABLE I. THE EFFECT OF 420 R. TOTAL BODY X-RADIATION UPON WEIGHT, TEMPERATURE, AND SEDIMENTATION RATE OF UNTREATED AND EXSANUINOTRANSFUSED DOGS
(Each Number Represents the Arithmetic Mean of 17 Animals)

DAYS AFTER X-RADIATION	BODY WEIGHT (KILOGRAM)		TEMPERATURE (° C.)		SEDIMENTATION RATE (MM. PER HOUR—UNCORRECTED)	
	UNTREATED	TRANSFUSED	UNTREATED	TRANSFUSED	UNTREATED	TRANSFUSED
0	6.8	7.5	39.4	39.5	17	18
1	6.5	7.4	39.0	39.2	26	17
2	6.3	6.8	39.1	39.0	24	26
3	6.4	6.9	38.9	39.1	27	38
4	6.2	6.7	39.2	39.6	29	38
5	6.3	6.8	39.5	40.1	25	43
6	5.9	6.3	40.4	40.1	43	48
7	5.4	5.9	40.3	40.4	47	49

TABLE II. THE EFFECT OF 420 R. TOTAL BODY X-RADIATION UPON WHITE BLOOD CELL COUNT, RED BLOOD CELL COUNT, AND HEMATOCRIT OF UNTREATED AND EXSANGUINOTRANSFUSED DOGS (Each Number Represents the Arithmetic Mean of 17 Animals)

DAYS AFTER X-RADIATION	WBC (THOUSANDS PER MM.^{3} OF BLOOD)		RBC (MILLIONS PER MM.^{3} OF BLOOD)		HEMATOCRIT (VOLUME OF CELLULAR ELEMENTS IN PER CENT OF TOTAL BLOOD VOLUME)	
	UNTREATED	TRANSFUSED	UNTREATED	TRANSFUSED	UNTREATED	TRANSFUSED
0	12.7	12.7	5.5	5.6	47	46
1	11.2	23.2	4.8	5.5	45	48
2	8.7	12.4	4.5	5.1	41	47
3	3.2	4.3	4.4	4.8	46	46
4	2.0	2.5	4.7	4.6	47	42
5	1.3	1.5	5.0	4.4	45	43
6	0.53	0.86	4.5	4.2	40	39
7	0.47	0.79	4.1	4.0	43	41

during either the latent period or the acute, terminal phase. None of the exsanguinotransfused dogs survived the second postirradiation week.

Laboratory Examination.—By regular determination of temperature and body weight, and by piloting hematological studies, the following results were obtained (Tables I and II): (a) As early as one day after exposure, a decrease in body weight became noticeable and continued steadily throughout the survival period. Treated and control animals paralleled each other. Significant in this respect were the petechial, purpuric, and frank hemorrhages found in the edematous alimentary tract post mortem. (b) Generally, the rectal temperature remained normal throughout the initial and latent periods. The approach of the acute, terminal phase was indicated by a sudden rise in temperature of all dogs, treated as well as controls. (c) Although a wide variation in sedimentation rate (from 1 to 60 mm. per hour) among the individual dogs was observed prior to the exposure and during the first postradiation days, values of more than 40 mm. per hour were obtained in all of the animals during the acute, terminal phase. (d) Following irradiation, the red blood cell count and the hematocrit of most of the dogs displayed a small declining trend. The sera of only 3 animals revealed a slight hemolysis during the first two days after exsanguinotransfusion. (e) In the control animals, the white blood cell count decreased continuously, whereas in the treated dogs the count exhibited a different course. As demonstrated by Table II, a marked leukocytosis occurred following blood replacement. This increase in white blood cells was noticeable throughout two days; it was observed in 16 of the 17 transfused dogs. Application of Student's t-test disclosed that the difference in the white blood count between control group and treated group was statistically highly significant the first day after irradiation. It was not significant the second day. Following the pronounced initial leukocytosis, the white blood cell count of the treated animals dropped rapidly to low values, which subsequently, paralleled those found in the controls. The nature and the origin of the white cells found during the leukocytosis phase were not determined. Since number and morphological appearance of the white cells in the donor blood remained normal throughout the twenty-four-hour storage period, approximately as many leukocytes were given as were removed. The donor cells, therefore, would not account for an absolute

increase; this would be particularly true if the viability of leukocytes were compromised by storage. Hence, the leukocytosis following transfusion appeared to be a reaction of the recipient's hemopoietic system.

Comparison of Survival Times.—As indicated by the distribution diagram of Fig. 3, a surprisingly uniform time course of radiation sickness was observed among the dogs inasmuch as 20 of the 34 irradiated animals (59 per cent) succumbed at the seventh and eighth day after irradiation. Of these 20 dogs, 9 were controls and 11 were transfused. The survival times of the remaining dogs were scattered between the third and twelfth day without any appreciable difference between controls and treated animals.

The statistical values for the survival times in days of the control group were: mean, 8.18; standard deviation, 2.13; standard error of mean, 0.52; 95 per cent confidence interval for means, 7.13 to 9.23 days. The corresponding data for the exsanguinotransfused group were: mean, 7.76; standard deviation, 1.98; standard error of mean, 0.48; 95 per cent confidence interval for means, 6.79 to 8.73 days. Hence, early exsanguinotransfusion does not significantly change the survival time following lethal total body x-radiation.

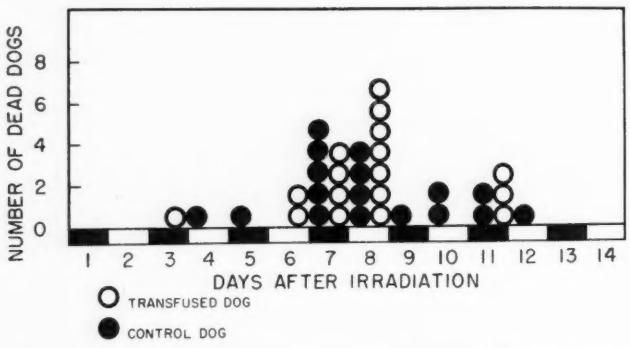

Fig. 3.—The distribution of the survival times of 34 dogs that received 420 r. total body x-radiation. Each solid circle represents the death of one of the 17 untreated control animals; each open circle, the death of one of the 17 exsanguinotransfused animals.

DISCUSSION

Cross transfusions[14] and small exchange transfusions, either alone,[15, 16] or combined with aureomycin therapy,[16] reportedly have proved beneficial in the experimental management of radiation sickness. These results, however, should not be overemphasized inasmuch as Allen* was not able to establish the therapeutic value of transfusions alone in dogs. The present experiments demonstrated that the clinical course was not appreciably altered by performing a single 90 per cent exsanguinotransfusion within three hours after exposure to a lethal dose of x-radiation. Three factors—transfusion procedure, incompatibility of blood, and dose of radiation might be responsible for this result.

In order to test the value of exsanguinotransfusion as a potential phase in the treatment of radiation sickness, no sham operations were performed upon

*Quoted in Hempelmann and co-workers.[17]

the controls. Thus, the treated group had to withstand the additional stress of prolonged anesthesia and a surgical operation. The complete recovery of most of the animals within a few hours disproves the importance of this factor.

It is difficult to assess thoroughly the influence exerted by minor incompatibilities of the blood. Although a single administration of incompatible blood usually does not provoke serious reactions in a normal dog,[12] it may, nevertheless, act as a further stress to the irradiated animal. However, the role played by this factor should not be overemphasized, since a clear beneficial effect has been reported of transfusions in which neither typing nor cross-matching of the prospective donors and recipients was performed.[14]

The severe clinical picture displayed by the irradiated dogs leads to the conclusion that the dose (420 r), administered under the conditions of the present experiments, is appreciably above the LD_{100} level. Probably, in this dose range no effect of transfusions upon the ultimate mortality can be expected[15] because the potential benefit of a blood exchange may be obscured by three processes. First, high amounts of x-radiation may directly affect the tissue so strongly that even a complete elimination of the indirect action does not appreciably change the clinical course. Second, although more than 90 per cent of the hypothetical noxious substances have been removed from the vascular system by an early exsanguinotransfusion, a continuous or continual formation of such agents may occur. Thus, in the high dose range, transfusions at different time intervals after exposure would be necessary to achieve a marked result. Third, the severe radiation insult inflicted upon the gastrointestinal tract by high doses of total body radiation may grossly compromise the entire nutrition of the dog. The progressive weight loss, observed in all of the irradiated animals of the present experiment, approximates that of complete starvation (Table I). Intense total body radiation is known to produce in the alimentary channel pronounced disorders, functional, and morphological, e.g., motility,[18] resorption,[19, 20] hemorrhages, ulceration, and edema[21, 22, 23]; hence an adequate enteral nutrition is precluded. Therefore, final judgment of exsanguinotransfusion as one possible phase in the treatment of radiation sickness must be held in abeyance until completion of further experiments combining blood exchange with parenteral feeding.

SUMMARY

An exsanguinotransfusion method for dogs has been developed that enables the replacement of more than 90 per cent of the recipient's blood by stored donor blood.

This procedure was employed in 20 dogs within two hours following exposure to a lethal dose (420 r.) of total body x-radiation. Clinical observations and laboratory examinations of these animals were compared with the findings obtained in 20 untreated control dogs exposed to an equal amount of irradiation.

No significant differences between the treated and the untreated groups were found in the general clinical course, in most of the laboratory studies, nor in survival time.

Following irradiation, a marked leukocytosis occurred in the transfused dogs throughout a period of two days, in sharp contrast to the leukopenia seen in the untreated control animals.

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BLOOD LIPOPROTEINS IN TRAUMATIC INJURY

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INTRODUCTION

IT WAS reported from this laboratory that twenty-four hours after acute local cold injury to a rabbit's hind leg, a massive hyperlipoproteinemia could be observed.[1] It was also reported that the extent of this increase was a rough measure of the degree of injury.[2] These data pose a number of very practical questions. Is the lipoprotein response specific for cold injury or will other trauma induce similar changes? Will repeated traumatic episodes maintain high lipoprotein levels in rabbits? Can increased lipid concentrations in the injured tissues be demonstrated? Will the hyperlipoproteinemia of traumatic injury respond to heparin administration in a manner similar to that reported by Graham[3] in cholesterol-fed rabbits and atherosclerotic human beings? Will adenosine-5-monophosphate modify the injury hyperlipemia, in accordance with previous reports from this laboratory, in normal[4] and atherosclerotic[5] chickens? Can tissue ischemia alone account for the increased blood lipoprotein levels recorded, or can a direct effect of trauma on muscle tissue protein be demonstrated? The series of experiments to be described were undertaken in an effort to satisfy such inquiry.

MATERIALS AND METHODS

Protocols for inflicting various types and degrees of experimental injury are described below. Parallel control rabbits were maintained for each group of experimental animals subjected to trauma. Except as specifically noted to the contrary, all animals were bled by cardiac puncture twenty-four hours after injury to the experimental groups. The analyses for plasma and tissue lipid and lipoprotein components as accomplished in this laboratory,[6, 7] along with assays for plasma calcium,[9] inorganic phosphorus,[10] potassium† and serum sodium† concentrations as may have been performed in each experiment are recorded in the appropriate table or figure. All rabbits utilized in this study were male albinos of the New Zealand strain, each animal weighing approximately 2 kilograms.

a. *Heat Injury (Mild).*—Rabbits were exposed to mild local heat injury of the right hind limb in a manner similar to that previously described for local cold injury,[1] except for the immersion of the condom-covered hind limb in a 50° C. water bath for five minutes with the animal under Pentothal anesthesia.‡ Twenty-four hours prior to exposure, 30 ml. of blood had been taken from each rabbit (control and experimental) by heart puncture with a lightly heparinized syringe. After injury the animals were returned to individual

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†Flame spectrophotometry.

‡Barbiturate anesthesia had been shown not to alter the response of the rabbits to acute local cold injury.[8]

TABLE I. THE EFFECTS OF HEAT INJURY ON THE CONCENTRATIONS OF PLASMA COMPONENTS. MEANS AND LEVELS OF SIGNIFICANT DIFFERENCE

		CHOLESTEROL (MG. %)			LIPOPROTEINS (MG. %)									PACKED CELL VOLUME (% CELLS)		
					S_f 0-12			S_f 12-20			S_f 20-100					
A. MILD INJURY	NO.	PRE-	POST-	Δ	PRE-	POST-	Δ	PRE-	POST-	Δ	PRE-	POST-	Δ	PRE-	POST-	Δ
Control	32	46	57	+11	33	28	–5	15	32	+17	26	87	+61	40	31	– 9
50° C. (5 min.)	30	47	66	+19	35	41	+6	19	49	+30	30	95	+65	41	31	–10
P**			<0.01			.02			.02			0.5			0.5	

B. SEVERE INJURY	NO.	CHOLESTEROL (MG. %)	LIPOPROTEINS (MG. %)			LIPID PHOSPHORUS (MG. %)	PACKED CELL VOLUME (% CELLS)	INORGANIC IONS (MEQ./L.)		
			S_f 0-12	S_f 12-20	S_f 20-100			CALCIUM	PHOSPHORUS	SODIUM
Control	27	50	19	11	10	4.46	43	7.45	3.21	146
50° C. (30 min.)	27	94	39	63	121	7.19	41	6.00	3.06	148
P*		<.001	<.001	<.001	<.001	<.001	<.01	<.01	.06	.06

P** = Probability that net change is due to chance (Mann-Whitney test).
P* = Probability that difference is due to chance (Mann-Whitney test).

Fig. 1.—Longitudinal sections of the tibialis anticus muscles of control and heat-injured rabbits. *A*, Control; *B*, mild injury; *C*, severe injury. (Hematoxylin and eosin; ×420.)

cages until twenty-four hours after exposure, when a second 30 ml. blood sample was taken from each rabbit. A small portion of blood was taken for determination of packed cell volume and the remainder centrifuged to yield unhemolyzed plasma. All plasma samples were analyzed for total cholesterol and the S_f 0-12, 12-20, and 20-100 classes of lipoproteins. The "pretraumatic" value was subtracted from the "posttraumatic" value for each rabbit and recorded as a positive or negative net difference. Mean net differences were then taken for each plasma variable and the significant level of the net effect of mild heat injury recorded in terms of "P." The results are shown in Table I, *A*.

b. *Heat Injury (Severe).*—Rabbits were treated exactly as described previously except that no blood samples were taken prior to exposure in order to prevent any possible effect of hemorrhage.* Further, the rabbit legs were immersed in a 50° C. water bath for thirty minutes instead of five minutes. Analytical results and levels of significance are shown in Table I, *B*. Since the tibialis anticus was the principal muscle involved in the injury, representative sections from the control, mild injury, and severe injury groups were cut for microscopic examination. These photomicrographs are shown in Fig. 1.

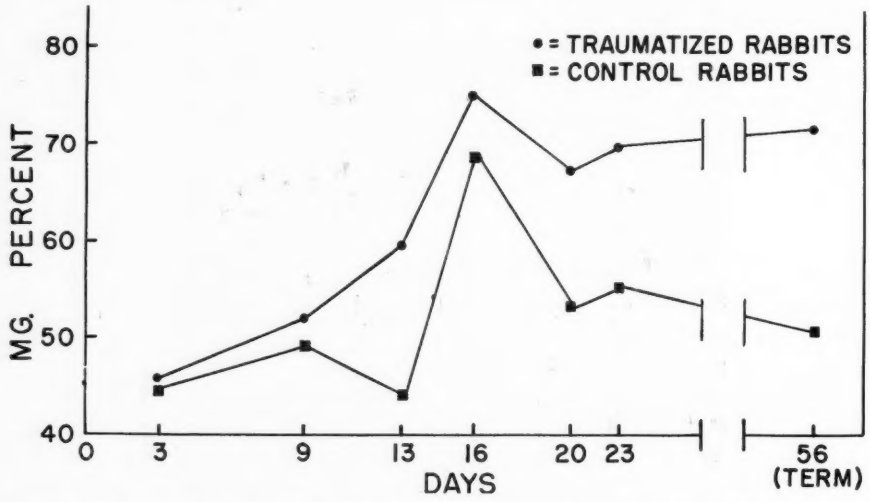

Fig. 2.—Time trend of serum cholesterol concentrations in control rabbits and rabbits subjected to repeated trauma.

c. *Repeated Traumatic Injury.*—Rabbits were subjected to periodic injections of 4 ml. of 100° C. physiologic saline on the first, seventh, tenth, fourteenth, seventeenth, twenty-first, and twenty-fourth days. The first injection was made into the right tibialis anticus muscle after Pentothal anesthesia. Blood specimens (3 ml.) from all animals were drawn from the ear vein for serum cholesterol determination on the fourth day and at each of the afore-mentioned times except the seventh day. The time trend of serum cholesterol concentrations is seen in Fig. 2. Since the control group seemed to be responding to the continued trauma of hemorrhage, the sampling procedure was stopped after twenty-four days, but injury to the experimental group continued twice weekly for a total of eight weeks. The injection was made on alternate sides into the tibialis anticus, gastrocnemius, quadriceps femoris, biceps-semimembranosa-tendinosa groups, gluteus, and the lateral vertebral muscle groups. The sites of injection frequently sloughed locally and became infected. No therapy to these areas was attempted. All injections were made under Pentothal anesthesia.

*When combined pre-post plasma lipid data on 80-odd control animals from several experiments were subjected to statistical analysis, a significant effect of hemorrhage from presampling was obtained.[5]

After eight weeks had elapsed, blood samples were taken from control and experimental groups for analysis. The animals were then sacrificed by a massive intravenous injection of Pentothal and the kidneys and aortas excised in toto and carefully cleaned of adherent fat and connective tissue. These organs were dried and analyzed for cholesterol and lipid phosphorus. The results of all terminal analyses are shown in Table II.

TABLE II. BLOOD AND TISSUE ANALYSES ON CONTROL RABBITS AND RABBITS SUBJECTED TO REPEATED INJURY—MEANS AND LEVELS OF SIGNIFICANT DIFFERENCE

	CONTROLS		INJURED		
	NUMBER	MEANS	NUMBER	MEANS	P*
Plasma lipid phosphorus and lipoproteins (mg. %)					
Lipid phosphorus	13	3.72	14	5.11	.015
S_f 0- 12	14	22	13	69	<0.01
S_f 12- 20	13	15	13	89	<0.0001
S_f 20-100	14	19	13	69	<0.001
Plasma ions (meq./L.)					
Sodium†	14	147.5	13	146.4	No significant difference
Potassium	13	4.83	13	3.89	<.001
Calcium	14	6.01	14	5.69	.06
Inorganic phosphate	14	2.51	14	2.29	.013
Aorta					
Cholesterol‡	14	4.8	14	4.3	No significant difference
Lipid phosphorus‡	14	0.68	14	0.56	.04
Per cent fat	10	15.67	9	14.69	No significant difference
Kidney					
Cholesterol‡	13	18.2	14	19.0	No significant difference
Lipid phosphorus‡	10	3.29	13	3.67	No significant difference
Per cent fat	14	26.34	10	26.99	No significant difference

*Probability that difference is due to chance (Mann-Whitney test).
†Analysis done on serum.
‡Milligram per gram dry weight.

d. *Crushing Injury.*—Rabbits to be subjected to crush injury were anesthetized with Pentothal Sodium (approximately 1 ml. of a 5 per cent solution) administered slowly into the ear vein until a suitable plane of anesthesia had been reached.* The right hind food was then carefully put through a spring-loaded hand wringer, of the type used in automobile service stations, until the rollers exerted pressure over the mid-calf region. An effort was made to keep the foot and leg in supination, but medial rotation invariably occurred as soon as the calcaneus cleared the rollers. Of the thirteen animal limbs crushed in this manner, only one fracture was seen. The injury was a comminuted simple fracture at the junction of the middle and lower third of the tibia, probably resulting from the leverage applied over the calcaneus as a fulcrum. This injury was splinted and the animal included in the series. In two other instances small areas of skin were avulsed. These areas were dressed with Sulfamylon ointment and the rabbits included in the experimental crush injury group. Tension on the rollers was adjusted so that the springs were just engaged when the wringer rollers were in apposition. No adjustment of pressure was made once the crushing procedure was begun, since all the animals were of approximately the same size and, thus, were subjected to essentially the same pressure. Control animals were also anesthetized and then returned to individual cages.

*The effect of anesthesia on the lipids and lipoproteins of normal and injured animals has been found to be nonsignificant.

Twenty-four hours following the injury, approximately 30 ml. of blood were taken from each animal for analyses. The right tibialis anticus muscle was removed from each animal after sacrifice with a fatal dose of Pentothal Sodium. The excised muscles were separately frozen in liquid air and reduced to granular form in a mortar. The ground muscles were individually lyophilized and the dry material was powdered in a mortar. The results of analyses for the various blood and tissue components studied are summarized in Table III.

TABLE III. PLASMA AND TISSUE ANALYSES ON CONTROL RABBITS AND RABBITS SUBJECTED TO CRUSHING INJURY—MEANS AND LEVELS OF SIGNIFICANT DIFFERENCE

	CONTROLS		INJURED		
	NUMBER	MEANS	NUMBER	MEANS	P*
Packed cell volume (per cent cells)	13	39.5	13	41.5	.04
Plasma					
Lipid phosphorus†	13	3.4	13	5.0	<.01
Lipoproteins†					
S_f 0- 12	10	21	13	53	<.001
S_f 12- 20	13	3	12	46	<.001
S_f 20-100	10	7	13	74	<.001
Cholesterol†	13	33	13	55	<.001
Ions					
Sodium‡§	13	146.0	13	147.4	0.1
Potassium‡	13	4.56	13	3.91	<.01
Calcium†	13	13.48	13	13.19	0.5
Inorganic phosphate†	13	6.37	13	5.98	.09
Muscle cholesterol‖	12	1.1	12	3.8	<.001

*Probability that difference is due to chance (Mann-Whitney test).
†Milligram per cent.
‡meq./L.
§Analysis done on serum.
‖Mg. per gram dry weight.

e. *Radiation Injury.*—The right hind limbs of rabbits from the knee distally were exposed to 30,000 r. of x-irradiation. As seen in Fig. 3, the entire animal was shielded with lead sheeting, with only the right hind limb exposed, and immobilized in this position over the x-ray beam for a period of thirty minutes. The control animals were similarly immobilized under lead sheeting except that radiation was not applied.

Twenty-four hours after injury, 30 ml. of blood were taken from each animal for analyses. Cholesterol assays were done on the excised tibialis anticus muscles of the right hind limbs of all rabbits.[6] The results of all determinations are shown in Table IV.

f. *Ischemia.*—Rabbits were subjected to ischemia of the right hind limb by securing a tourniquet around the thigh with a hemostat. Cessation of blood flow in the region distal to the tourniquet was established by the failure of superficial veins to refill. In the experimental Group b rabbits, the tourniquet was allowed to remain in place for a period of thirty minutes. In the experimental Group c rabbits, the tourniquet was allowed to remain in place for a period of three hours. Plasma lipid and lipoprotein components were determined and the results are shown in Fig. 4.

g. *The Effects of Heparin and Adenosine-5-Monophosphate (AMP).**—The AMP used in this experiment was supplied in 10 ml. vials of gelatin suspension containing 284 mg. of AMP per milliliter. The AMP placebo was supplied in identical form by the same company and consisted of the gelatin carrier alone. Heparin repository (Lederle), 20,000 units per milliliter, in a menstruum carrier was employed. The menstruum carrier alone served as the heparin placebo. All drugs and placebos were administered subcutaneously. Hyperlipoproteinemia (HLP) was induced in rabbits by acute, severe local cold injury to the right hind limb, as previously described.[1] The rabbits were divided into eight equal

*Ernst Bischoff Company, Ivoryton, Conn.

TABLE IV. PLASMA AND TISSUE ANALYSES IN CONTROL RABBITS AND RABBITS SUBJECTED TO HIND LIMB IRRADIATION OF 30,000 r.

	NO.	CHOLESTEROL (MG. %)	LIPID PHOSPHORUS	LIPOPROTEINS (MG. %)			PACKED CELL VOLUME (% CELLS)	INORGANIC IONS (MG. %)		MUSCLE CHOLESTEROL (MG./GM. DRY WEIGHT)
				S_f 0-12	S_f 12-20	S_f 20-100		CALCIUM	PHOSPHORUS	
Control	15	42.3	3.9	18	12	19	44	13.4	6.25	2.86
Irradiated	15	83.8	7.2	58	86	95	43	11.3	5.96	4.40
P*		<.01	<.01	<.01	<.01	<.01	NSD†	<.01	NSD†	.02

*Mann-Whitney test.
†NSD = No significant difference.

groups and were treated over an eight-day experimental period. All animals were sacrificed on the ninth day, when blood was obtained for subsequent analyses. The schedule of treatments and analytical results are shown in Table V. Significance levels recorded thereon were determined by use of the Wilcoxen (Mann-Whitney) test.

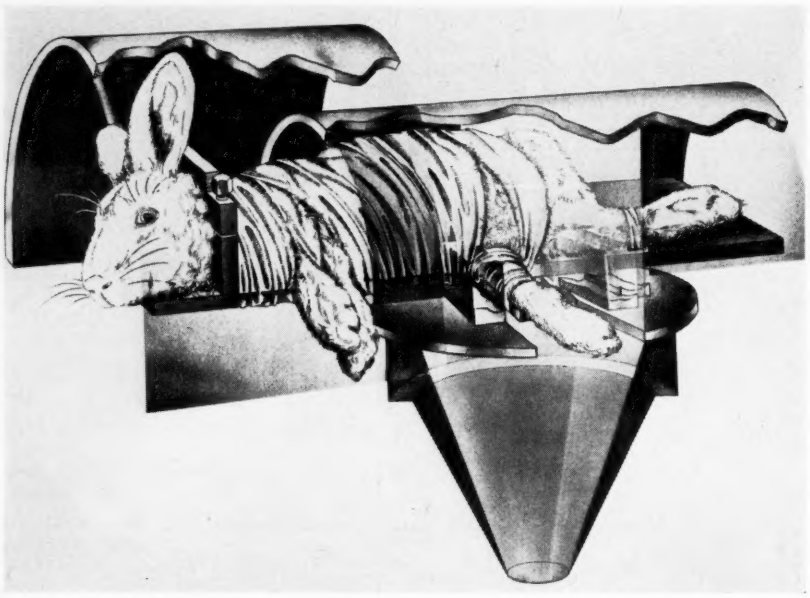

Fig. 3.—The rabbit immobilized and shielded for x-irradiation exposure of the right hind limb.

TABLE V. MEANS OF BLOOD PLASMA COMPONENTS AFTER THE ADMINISTRATION OF ADENOSINE-5-MONOPHOSPHATE (AMP), HEPARIN, AND THE TWO DRUGS TOGETHER TO NORMOLIPOPROTEINEMIC RABBITS AND IN RABBITS RENDERED HYPERLIPOPROTEINEMIC BY LOCAL COLD INJURY

	NUMBER	CHOLESTEROL (MG. %)	LIPID PHOSPHORUS (MG. %)	LIPOPROTEINS (MG. %)		
				S_f 0-12	S_f 12-20	S_f 20-100
Normolipoproteinemic						
Controls	20	60	4.1	24	14	17
AMP	19	50*	3.4†	18	5*	10*
Heparin	19	47	3.1†	29	4†	4†
AMP and heparin	19	48*	2.7†	29	2†	1†
Hyperlipoproteinemic						
Controls	18	101	6.0	76	64	80
AMP	18	118*	8.1*	70	66	181
Heparin	16	103	6.0	108*	26†	17†
AMP and heparin	15	110	6.7	127†	30†	24†

*Significantly different from control value at a level of P = .05 or less (Mann-Whitney test).

†Significantly different from control value at a level of P = .01 or less (Mann-Whitney test).

RESULTS AND DISCUSSION

There seems to be little doubt that acute local injury will induce marked blood lipid and lipoprotein increases in rabbits regardless of the type of physical trauma involved (Tables I-IV and Fig. 4). Furthermore, as seen

from the results of two levels of heat injury (Table I), the extent of the blood lipoprotein response is a rough function of the degree of injury. When the rabbit leg was exposed to 50° C. for only five minutes, lipid changes, although significant, were small and the S_f 20-100 class of lipoproteins failed to increase significantly. On the other hand, the more severe heat injury induced a massive hyperlipoproteinemia. As demonstrated by the significant decrease in

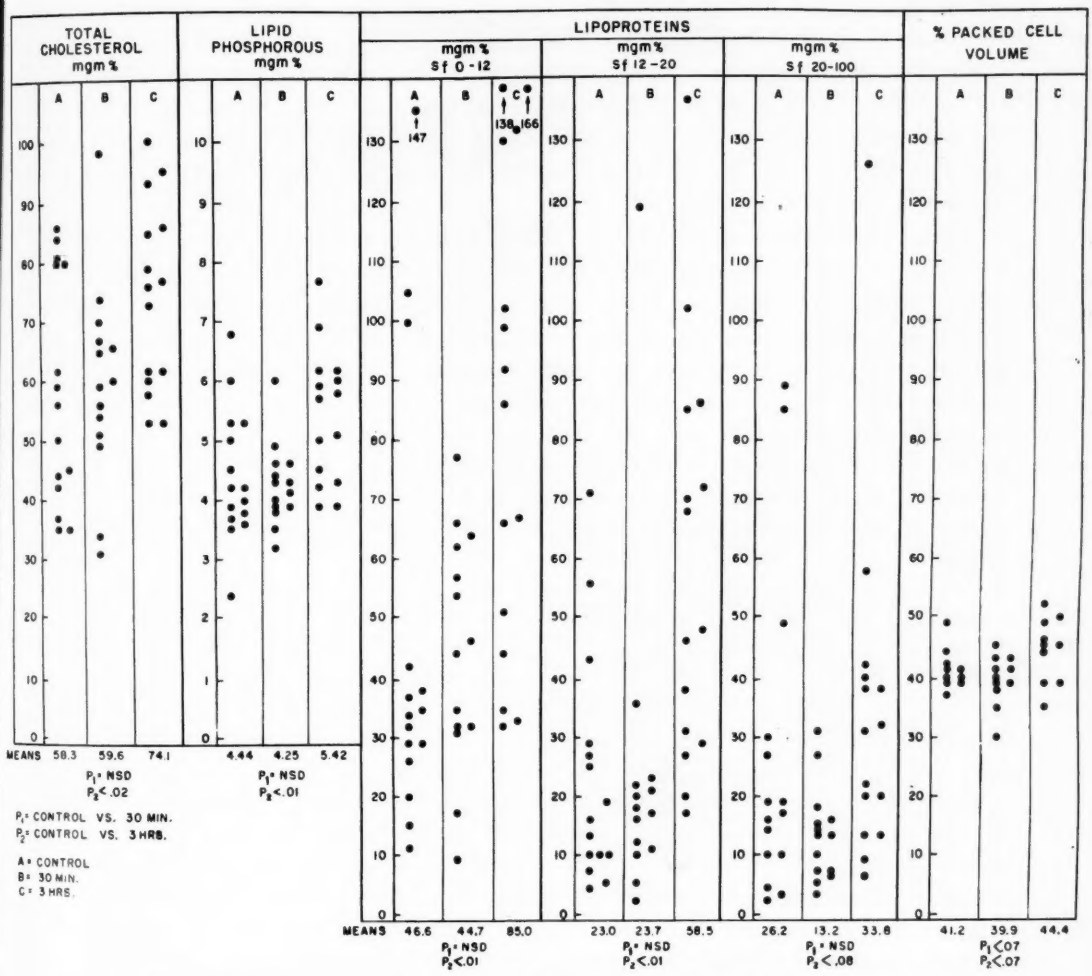

Fig. 4.—Blood plasma analyses in control rabbits and rabbits subjected to two levels of ischemia of the right hind limb—individual values, means, and levels of significant difference.

plasma calcium concentration and the trend toward lower levels of inorganic phosphorus, the effect is not merely one of hemoconcentration. Similar results are recorded after crushing injury, x-irradiation, and ischemia. Little can be said concerning the most effective type of trauma in raising plasma lipoprotein concentrations. The data would indicate that the response was greatest after severe heat injury, but the large variation within control groups would render such a conclusion most hazardous.

The data recorded in Table II demonstrate that repeated local injury will result in a relatively high maintenance level of blood lipids and lipoproteins in rabbits. Confirming the finding after heat injury by exposure, the results further indicate that the trauma of hot water injections will also result in decreased levels of certain inorganic ions. Surprisingly enough, in spite of greatly increased blood lipid concentrations over the eight-week period, analyses of aortal and kidney tissues at term failed to demonstrate changes. A decrease of doubtful significance was found in the aortal phospholipid concentration after repeated injury, but other lipid variables remained at control levels. These results cannot be considered definitive, however, because the injured animals lost considerable weight during the experimental period and it might be expected that any tissue lipid deposition would be offset by losses due to energy requirements.

Muscle data recorded in Table IV indicate that the high blood lipoprotein levels after injury are accompanied by increased concentrations of cholesterol in the traumatized tissues. The change had been observed after cold and heat injury (and is recorded here after crush trauma) but it was not reported because extensive bleeding into the tissue interstitial spaces under such circumstances might have accounted for the increased cholesterol concentrations. X-irradiation, however, does not challenge the security of the local vascular network and essentially bloodless samples can be obtained for analysis. The finding lends some support to the possibility that the hyperlipoproteinemia of injury is due, in part, to the increased synthesis of cholesterol in the injured tissue.

After lyophilized muscle tissues of control and irradiated rabbits had been analyzed for cholesterol, the remainder of the tissue samples were separately extracted with 0.05 M of Veronal buffer and subjected to paper electrophoresis partition.* Equal protein concentrations of a 0.05 ml. control sample and 0.05 ml. of an experimental sample were spotted side by side on a single paper strip and the electrical field applied. After eight hours the spots were developed with bromphenol blue and acetic acid. A sample electropherogram is shown in Fig. 5. It is established by inspection that x-irradiation has altered the electrophoretic characteristics of the muscle protein molecule. The increased mobility of a larger portion of the injured muscle protein may indicate a change of shape in view of a report by Barbu and Joly[11] that the more globular proteins have higher electrophoretic mobilities on paper.

The data in Table IV and Fig. 4 are completely consistent in that they demonstrate that tissue ischemia is not primarily involved in the hyperlipoproteinemia of injury. Ischemia remained a possible cause as long as the lipoprotein response was confined to trauma by heat, cold, or crush wherein the muscle blood supply suffered more or less complete destruction. The irradiated rabbits, however, developed the hyperlipoproteinemia after thirty

*Electrophoresis was accomplished at room temperature on Whatman No. 3 filter paper in locally constructed equipment patterend after that manufactured by the Precision Scientific Company. At no time during electrophoresis did the temperature in the chamber exceed 35° C. The paper was blotted dry before staining and the presence of proteins or polypeptides was established on the basis of positive biuret tests.

minutes of exposure, despite the maintenance of normal muscle blood supply. The experimental Group b rabbits, after thirty minutes of muscle ischemia, on the other hand, showed no significant change in blood lipoprotein levels. It would seem that an irreversible alteration of muscle protein, such as may have been demonstrated by paper electrophoresis, is a necessary prerequisite to the lipoprotein response.

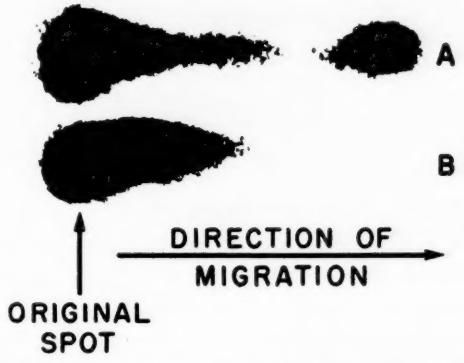

Fig. 5.—Paper electropherogram of a Veronal buffer extract of injured muscle protein (*A*) and control muscle protein (*B*). pH 8.6, ionic strength 0.055, 600 v., 15 Ma., 28,000 seconds.

The data in Table V establish that AMP administration will affect blood lipid and lipoprotein concentrations in rabbits as well as chickens, and that heparin will alter the hyperlipoproteinemia of injury in a manner similar to that reported for cholesterol-fed rabbits and atherosclerotic human beings by Graham.[3] With respect to AMP, the effects seem confined to the normolipemic state. Eight days of administration of AMP failed to reduce the high blood lipid and lipoprotein concentrations in the cold-injured rabbits. It should be remembered, however, that AMP has, thus far, been effective only if administered for an extended period (seven to fourteen days). The control animals in this experiment were given eight injections of AMP between zero time and sacrifice. Only a single injection of AMP was given to the injured group of rabbits after the induction of hyperlipoproteinemia. It remains to be determined if extended AMP administration will reverse the lipoprotein response in traumatized rabbits.

SUMMARY

Rabbits were separately subjected to local injury by heat, crushing, ischemia, and radiation. It was demonstrated that blood plasma lipoprotein concentration was markedly increased by each type of traumatic episode. There was an indication that lowered plasma calcium ion levels also followed traumatic injury. Repeated injury to the same animal over an eight-week period was associated with prolonged hyperlipoproteinemia in rabbits. The lipoprotein response to trauma could not be accounted for on the basis of tissue ischemia but the increased plasma lipoprotein levels were found to be associated with a concomitant increase of cholesterol concentration in the injured tissue.

Heparin administration to injured rabbits markedly altered the qualitative pattern of hyperlipoproteinemia, resulting in a marked shift toward lipoproteins of highest density. No effect of AMP on the hyperlipoproteinemia of traumatic injury could be recorded, but eight days of AMP administration to normolipemic rabbits decreased significantly the plasma lipid and lipoprotein components measured.

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METABOLIC OBSERVATIONS IN INSULIN-RESISTANT DIABETICS GIVEN INTRAVENOUS FRUCTOSE

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INTRODUCTION

PATIENTS with diabetes mellitus utilize fructose in much the same manner as do nondiabetics.[1] Reported experience of fructose administration in diabetics with insulin resistance has been limited.[2] Insulin resistance has been associated with a variety of disease entities, but the physiologic mechanisms responsible for insulin resistance have not been clarified.[3] Experiments were designed to evaluate whether the metabolic defect of insulin resistance is at the level of cellular penetration and initial phosphorylation of glucose as in the usual diabetic[4] or whether it involves some subsequent stage of carbohydrate metabolism. Changes in the level of blood pyruvic acid were studied during and following intravenous administration of fructose to two diabetic patients who were resistant to insulin.

MATERIALS AND METHODS

Two patients with insulin resistance were available for study. Case 1, C. B., a woman, aged 53 years, had had diabetes for five years, was insulin resistant for a month prior to study, and was receiving 600 units of crystalline insulin daily at the beginning of the studies and 1,000 units daily four months later at the time of her death. She had an unexplained anemia and cirrhosis of the liver. Case 2, N. G., a man, aged 67 years, had had diabetes for two years, was insulin resistant for three weeks prior to study, and was given 350 to 600 units of insulin daily. He has remained insulin resistant up to the time of this writing five months after initiation of the studies. There were no associated diseases. Both patients were fasting and at bed rest overnight prior to each observation. The last dose of crystalline insulin was given not later than the afternoon before the observations were made. Long-acting insulin was not given during the observations. Fructose,* 100 Gm., was given in a 10 per cent solution over approximately a two-hour period. Glucose was administered in a similar manner.

Additional control observations in Case 1 consisted of hourly specimens of blood for sugar, potassium, inorganic phosphorus, calcium, and pyruvic acid, before, during, and following a meal which was composed of carbohydrate, 40 Gm.; protein, 20 Gm.; and fat, 15 Gm.; a meal which was composed of carbohydrate, 45 Gm.; protein, 20 Gm.; and fat, 15 Gm.; plus crystalline insulin, 60 units subcutaneously; crystalline insulin, 60 units subcutaneously without food; and crystalline insulin, 1.0 unit per kilogram of body weight intravenously.

All determinations were made in duplicate and the average of the two was used as the final value. Venous blood for pyruvic acid determinations was collected without stasis and immediately deproteinized with trichloracetic acid. Serum for potassium, phosphorus, and

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*The fructose was supplied by Mead Johnson & Company.

calcium determinations was separated from the clot within thirty minutes after being drawn except for some samples of Case 1 in which full clotting had not taken place for forty-five minutes.

Blood sugars were determined by the Somogyi-Nelson method,[5] phosphorus by a slightly modified Fiske-Subbarow method,[6] and calcium by the V method.[7] Serum potassium determinations were made on a Baird flame photometer using the internal standard procedure. Pyruvic acid levels were determined by a modification of the Friedemann-Haugen method.[8] The modifications were as follows: the protein-free centrifugate (before the addition of 2, 4-dinitrophenylhydrazine) was placed in a boiling water bath for five minutes in order to destroy any acetoacetic acid that may have been present[9]; the organic solvent used to extract the phenylhydrazone was a xylene, n-butanol mixture (75:25 volume); fifteen seconds of vigorous shaking of the test tube stoppered with a synthetic rubber stopper was substituted for bubbled air.

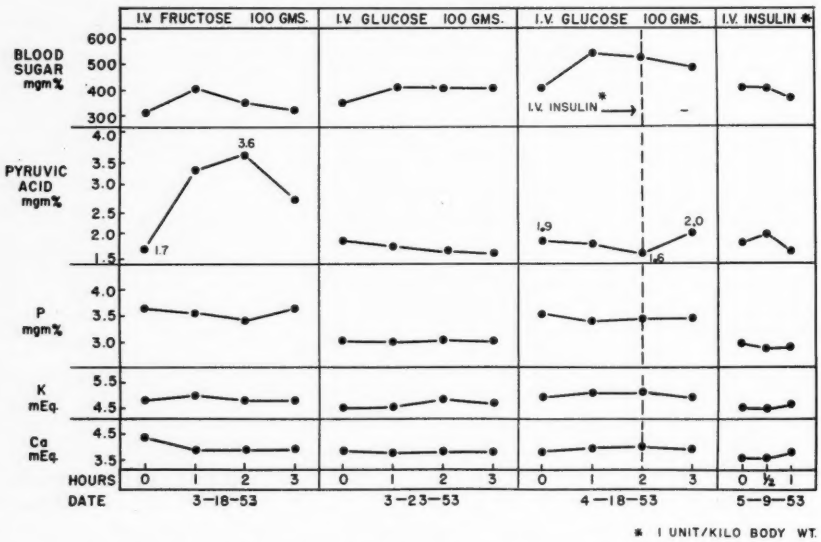

Fig. 1.—C. B., aged 53 years. Insulin resistance. Response to fructose, glucose, and insulin.

RESULTS

Case 1 (C. B.).—A rise in pyruvic acid levels from 1.7 to 3.6 mg. per cent occurred during fructose administration. Glucose alone did not alter the pyruvic acid levels. During control observations, the pyruvic acid levels remained constant. Base-line pyruvic acid levels were elevated in all experiments. At the termination of a glucose infusion, 1.0 unit per kilogram of body weight of crystalline insulin was given intravenously and was followed by a very slight rise in pyruvic acid. Potassium, phosphorus, and calcium levels were not significantly altered in the control observations or by intravenous glucose and fructose. Blood sugar levels rose to varying degrees in all experiments except when insulin alone was given.

Case 2 (N. G.).—As in the previous patient, fructose produced a significantly greater rise in pyruvic acid levels than did glucose alone or a glucose infusion followed by intravenous insulin. A consistent rise in pyruvic acid levels accompanied each fructose infusion given for five consecutive days. The order

of magnitude of this rise was as great with the fifth infusion as it was with the first. A very slight rise in pyruvic acid levels occurred with intravenous glucose. Intravenous insulin (0.35 unit per kilogram of body weight) following glucose administration resulted in a very slight rise in pyruvic acid levels. Base-line pyruvic acid levels were elevated in all experiments. Phosphorus levels consistently fell with each fructose infusion. In one instance there was a fall in phosphorus levels with glucose. Potassium and calcium levels were not altered by either glucose or fructose. Blood sugar levels rose with each infusion.

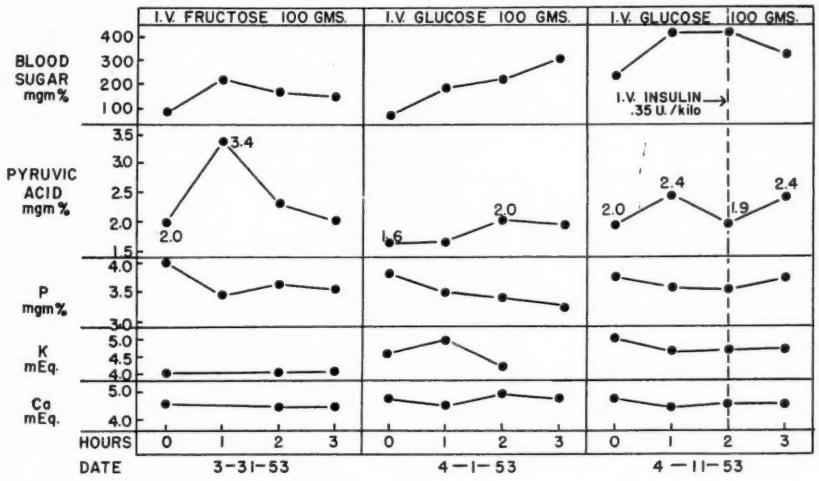

Fig. 2.—N. G., aged 67 years. Insulin resistance. Response to fructose, glucose, and insulin.

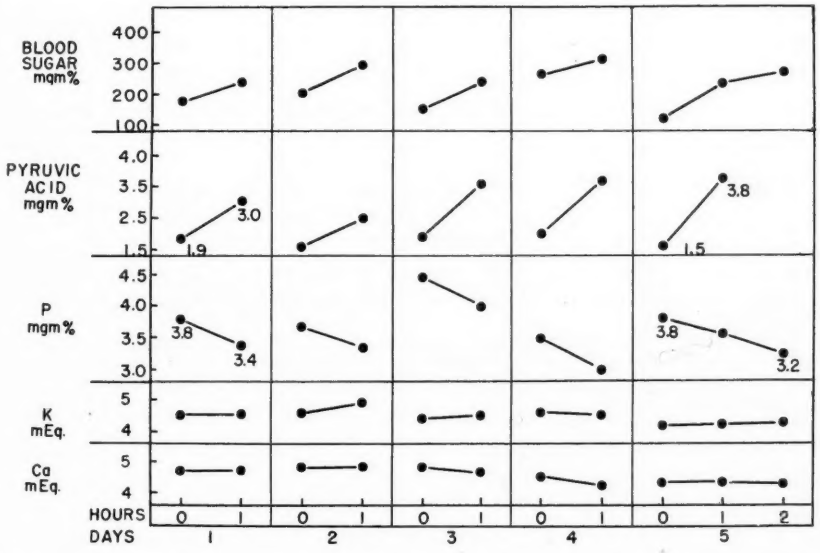

Fig. 3.—N. G., aged 67 years. Insulin resistance. Response to intravenous fructose (100 Gm.),

During the administration of fructose for five consecutive days, the insulin requirement fell markedly in Patient N. G., from 495 units to 80 to 160 units daily. Upon discontinuing fructose, it was necessary to return to insulin doses of over 300 units daily.

DISCUSSION

Fructose may enter the Meyerhof-Embden system via the formation of fructose-6-phosphate and from there may be converted to glycogen through glucose-6-phosphate and glucose-1-phosphate. An alternate pathway is the conversion of fructose-6-phosphate to fructose-1:6-diphosphate, then into 3-carbon compounds and to pyruvic acid.[10] Diabetic as well as normal persons respond to a fructose load by an elevation in blood pyruvic acid and fall in phosphorus levels.[1] The response in pyruvic acid and phosphorus accompanying a glucose load in the diabetic is delayed or minimal.[1, 11] In both patients studied the pyruvic acid response to fructose was prompt and marked. In no other circumstances (food, glucose, insulin, and glucose plus insulin) was the response of the same order of magnitude. This rise in pyruvic acid suggests that in insulin resistance the defect does not lie in the pathway of the conversion of fructose to pyruvic acid. This would indicate that the insulin-resistant diabetic does not qualitatively differ from the usual diabetic in the conversion of fructose to pyruvic acid.

During the period of the chronic administration of fructose in Patient N. G., the insulin requirement decreased. The 100 Gm. of fructose represented approximately one-third of his daily carbohydrate intake. Insulin does not influence fructose utilization when the latter is given intravenously.[12, 13] This may account for the decreased insulin requirement during this period.

SUMMARY

Two cases of insulin resistance were given infusions of fructose. Elevation in the pyruvic acid levels was similar to that expected in the noninsulin-resistant diabetic and the nondiabetic. This suggests that there was no defect in the metabolic pathway of fructose to pyruvic acid in the insulin-resistant diabetics studied.

The technical procedures were carried out by Patricia Megan.

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STUDIES ON PRIMARY AMINES

II. Their Natural Occurrence in Urine of Normotensive and Hypertensive Subjects

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PRESENT information on naturally occurring primary amines in mammals includes reports of the presence of hydroxytyramine,[1] norepinephrine,[2] histamine,[3] ethanolamine,[4] methylamine,[5] and controversial evidence for the presence of isoamylamine.[6, 7] These compounds have been identified by pharmacologic or chemical methods. Reports of separation of primary amines as a class of compounds have not been seen. Decarboxylation of amino acids without deamination would lead to formation of the corresponding primary amines, most of which are known to have pressor activity. The extent to which such a reaction occurs is not known.

Studies[8-10] have been made during the past few years on many primary amines with particular emphasis on those of biologic interest, the decarboxylated amino acids. Methods developed for separation, partial identification, and quantitation have been published.[9] In this paper the methods of extraction of amines from urine will be presented together with results indicating the number, the variability, and an estimate of the amount of these compounds excreted daily by adults.

METHOD

The following two methods are satisfactory for extraction of the amines from urine. The chromatographic and quantitative methods[9] will be referred to only as they apply to urine. One hundred and twenty-five twenty-four-hour urine samples from 36 subjects were analyzed. Twelve subjects were normal, 20 had arterial hypertension, and 4 suffered from other disorders.

Batch Extraction Method.—Three hundred milliliters of a twenty-four-hour urine sample collected in a bottle containing 50 ml. toluene and 10 ml. of 2.4 N HCl are brought to pH 3.5 ± 0.5 with glacial acetic acid. One hundred milliliters of chloroform are added. After shaking on a mechanical shaker for ten minutes, the mixture is allowed to stand until the emulsion is broken. The chloroform layer is discarded. Five-sixths of the aqueous layer is adjusted to a pH of 10.0 ± 0.5 with 10 ml. of hot saturated sodium borate and concentrated sodium hydroxide. (The borate buffer may be eliminated without materially affecting the results.) Fifty milliliters of chloroform are added and the mixture is shaken for fifteen minutes on a mechanical shaker. The emulsion is broken by centrifugation and the chloroform layer removed by separatory funnel. The amines may now be extracted

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back into 5 ml. of 2.4 N HCl and concentrated to dryness in vacuo at room temperature. By this method recovery is poor for the amount of labor involved, but several samples can be run simultaneously.

Continuous Extraction Method.—Twenty-four-hour samples of urine collected under toluene and 10 ml. of 2.4 N HCl are brought to pH 10.5 to 11.5 with sodium hydroxide, centrifuged, and the supernatant extracted for eight to twelve hours with carbon tetrachloride in a Wolf-Hirshberg continuous extractor. The solvent is cycled by distillation from a flask containing 50 ml. of 2.4 N HCl through a sintered disc in the top of a cylinder containing approximately 840 ml. of the alkaline urine. The greater part of the recoverable amines are obtained in twelve hours, but small quantities are still being extracted after twenty to twenty-four hours.* The acid layer of the extract is extracted three times with equal volumes of petroleum ether. The ether fractions are discarded. The acid layer is concentrated to dryness in vacuo at $<40°$ C. The advantage of this method is that fairly large volumes can be extracted with a minimum of labor and maximum yields. Its limitation is in the over-all time required for a single extraction.

Chromatography.—The dried residue is taken up in 2 ml. of 1:1 water-isopropanol and filtered. The filter paper is rinsed with three 1.0 ml. portions of water-isopropanol. The filtrate is diluted so that 0.5 ml. represents 5 per cent of the original urine, and mixed. One-half milliliter is added to a mixture of 0.1 ml. 0.01 M methylamine hydrochloride, 0.05 ml. 0.01 M ethylamine hydrochloride, and 0.05 ml. 0.01 M isoamylamine hydrochloride. This and a mixture of the known amines without added extract are applied to one-inch strips of a sheet of No. 1 Whatman filter paper. The remainder of the extract is applied 0.5 ml. per inch to another strip on the same chromatogram. It is then run by the ascending technique of Williams and Kirby[11] for eighteen to twenty-four hours in a butanol-water solvent. Alternatively, a solvent containing 20 per cent distilled water, 30 per cent butanol, and 50 per cent isopropanol is useful for separating hydrophilic amines with low Rf values. Three-fourths of the chromatographed extract are left unsprayed for elution followed by assays and special tests. The position of the known amines, with and without extract added, is compared with the spots of the extract. The Rf values of the known amines may be modified by the presence of urine extracts as well as by other known amines. Chemical characteristics and sometimes identity are suggested by the Rf value, the color of the spots, and the rates of color development. The ratio of the ninhydrin-amine optical densities at 405 and 570 mμ was determined for each unknown eluate as previously described.†[9]

Procedure for Mannitol Hexanitrate Study.—Patients were selected from the Hypertension Clinic of the Washington University Clinics. All but one (N. H.), who had anhidrosis, were hypertensive. Two were men; nine were women. After several preliminary tests a procedure for studying the effect of mannitol hexanitrate (Nitranitol)‡ on the excretory pattern of primary amines was developed. The patient was kept on the same diet to which he had been accustomed, in many cases a special low-calorie diet prescribed by the Washington University Food Clinic with specified quantities of protein, carbohydrate, fat, and salt. However, no attempt was made to record how well the patients adhered to their diets. Each patient was instructed how to collect a twenty-four-hour urine sample and was given a mimeographed sheet to record the times of beginning and ending each collection. Samples were kept in a cold room at 5° C. or in a freezer until they were extracted.

Half of the patients were given placebo for two days during which they collected two twenty-four-hour samples of urine, were then placed on Nitranitol and, after at least

*For example, in the first twelve hours of extraction 48.5 per cent of added isoamylamine was recovered from a urine sample which had been previously extracted until free of amines. In the second twelve hours only 3.5 per cent could be recovered. Whether the major loss is by destruction or volatilization of the amine during the extraction process is not known.

†Originally 400 and 570 mμ optical densities were read on water eluates from chromatograms. A solvent of 1:1 water-isopropanol has been found better for removing ring-substituted amines quantitatively from paper. With this solvent 405 mμ gives more precise readings than 400 mμ.

‡Nitranitol is the trade mark of the William S. Merrell Company's preparation of mannitol hexanitrate and is the preparation generously supplied by the Merrell Company for this study.

one day, collected two additional samples. The other half reversed the procedure, taking Nitranitol first and allowing at least four days to elapse while taking placebo before collecting two more twenty-four-hour samples. The placebo tablets resembled the Nitranitol tablets in every respect.

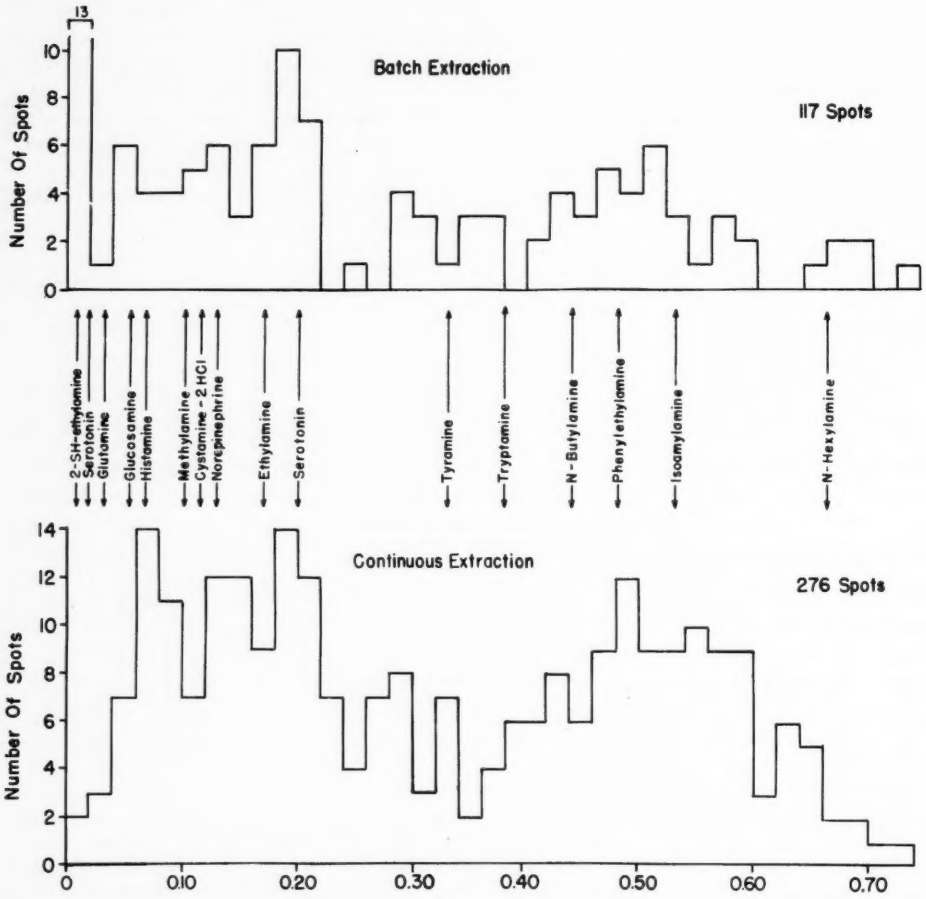

Fig. 1.—Frequency diagram of Rf values of primary amines extracted from urine by batch and continuous methods. Abscissa represents Rf values. Isoamylamine was run on each sheet, its Rf being used to correct observed values of both knowns and unknowns to a standard isoamylamine Rf of 0.53. Butanol saturated with water was the solvent. Rf's of 15 known primary amines are shown but do not identify any of the amines found in urine.

RESULTS

Number of Primary Amines in Human Urine.—As many as eight amines have been found in single twenty-four-hour samples of pathologic urine. In normal urines using the less efficient batch extraction method, as many as six amines have been found. Amines from all sources group themselves around 10 Rf peaks. The frequency profile of amines by Rf values for the two methods of extraction discussed is shown in Fig. 1, together with the Rf values of selected known primary amines. The variations in Rf peaks which occur when different methods are used may be explained by differences

in concentration of foreign extractives. Allowing for this variation, agreement by the different methods of extraction is good. The Rf ranges, though somewhat arbitrary, are comparable in the comparisons made.

When continuous extraction procedures were used, the number of free primary amines per pooled urine sample varied from 4 to 10. The variations may be due to conjugation, failure of formation, or destruction of the amines.

Batch Extraction.—Normal Urine: Table I compares the Rf ranges of individual extracts of normal male and female urine obtained by the batch extraction method. Variation in amine excretion between individuals is large. No sex difference which can be considered significant is apparent. No amine occurs in the urine of members of one sex which is never found in that of the other. The amines most frequently appearing in the urine of normal subjects under normal conditions have mean Rf's of 0.05, 0.14, and 0.48 in butanol-water solvent.

TABLE I. PRIMARY AMINES FOUND BY BATCH EXTRACTION IN NORMAL SUBJECTS

SUBJECT	NO. OF AMINES	Rf RANGES A 0.00	B 0.10	C 0.18	D 0.26	E 0.30	F 0.36	G 0.44	H 0.52	I 0.60	J 0.70
					Men						
D. F. D.	4	x	x			x		x			
P. H. D.	4	x	x		x			x			
H. M. C.	6	x	x		x	x		x	x		
E. T.	5	x		x		x	x	x			
H. M. P.	5	x	x			x	x				
H. A. S.	4		x	x	x			x			
Total Men (N = 6)	28	5	5	2	3	4	2	5	1		
Mean Men	4.7										
					Women						
E. F. P.	2		x					x			
L. W. S.	5	x	x			x		x	x		
A. H. D.	5	x	x	x			x	x			
E. D.	4	x		x		x		x			
G. H. C.	4		x	x	x			x			
M. T.	6	x	x	x	x		x		x		
Total Women (N = 6)	26	4	5	4	2	2	2	5	2		
Mean Women	4.3										
Total Normal (N = 12)	54	9	10	6	5	6	4	10	3		
Mean Normal per day	4.5	0.75	0.83	0.50	0.42	0.50	0.33	0.83	0.25		

Variations in Amine Profile on Two Consecutive Days.—While no exhaustive study has been made of the effect of exogenous factors on the excretion pattern of primary amines, it seems clear that they are important. There are both qualitative and quantitative daily variations in subjects on a normal diet and in patients on a regular hospital diet. While the batch method as applied cannot be assumed to extract total amines, the variability in those which appear indicate that one or two quite regularly occur in any one individual with others appearing at less frequent intervals. Table II shows the variability in the Rf pattern which was found in 12 pairs of twenty-four-hour urine collec-

TABLE II. DAY-TO-DAY VARIABILITY OF AMINES BY BATCH EXTRACTION ON TWO CONSECUTIVE DAYS

SUBJECT	DAY	PER CENT OF 24-HR. SAMPLE	Rf RANGES A 0.0	B 0.10	C 0.18	D 0.26	E 0.30	F 0.36	G 0.44	H 0.52	I 0.60	J 0.70	TOTAL
Normotensives													
D. F. D.	1	18.7	x	x	x					x			4
	2	14.2	x	x	x				x				4
H. A. S.	1	12.2		x	x	x			x				4
	2	15.0		x	x	x			x				4
E. F. P.	1	32.9			x	x			x				3
	2	29.8			x				x				2
H. M. P.	1	22.7	x	x	x				x				4
	2	21.7		x	x				x				3
N. H.	1	10.2	x						x				2
	2	8.93	x	x			x						3
Mean (N = 10)													3.3
Hypertensives													
L. S.	1	12.2		x		x		x	x		x		5
	2	15.8	x		x						x		3
L. S.	1	17.1								x			1
	2	18.6			x				x	x	x		4
L. Ro.	1	12.4			x				x		x		3
	2	16.1	x		x								2
L. Ro.	1	14.3			x				x			x	3
	2	14.2	x	x						x			3
E. W.	1	16.6	x	x				x	x				4
	2	25.6	x										1
E. W. .	1	16.2		x					x	x			3
	2	15.6	x	x				x	x				4
R. S.	1	10.8		x	x								2
	2	13.7	x	x	x								3
Mean (N = 14)													2.9

tions in nine subjects, four with hypertension. No attempt was made to control the diet, but conditions were otherwise constant for each of the paired collections. Qualitative differences between the amine profiles on subsequent days appear; the possibility that this is due in part to amines being present in minute amounts not measurable by the method has not been ruled out. In most cases the fraction of the sample extracted for each of the two days was approximately the same. Amines were classified in Rf ranges strictly according to Rf after correction to that of isoamylamine which was run as a control on each chromatogram. Nevertheless, it may be assumed that the overlap of Rf ranges of separate amines would occasionally place the same compound in two Rf categories. This shift may have occurred, for example, for amines of E. W. from Rf ranges f and g to g and h on the first day of the second pair of samples. In the second pair of L. R., none of the apparent amines were duplicated, although three of the amines had counterparts in the first pair. Ranges e and j showed only one amine each out of 24 twenty-four-hour urines. The greatest concentration of amines was found in the lower Rf bands and in the g (Rf 0.44-0.52) category.

Hypertension.—Because of the great variability of the amine profile between individuals, only striking and consistent differences can be considered significant in any single amine category. A much larger series would be

necessary to explore statistically the differences in frequency of occurrence of any amine. Inspection of the data shows that after batch extraction of hypertensive urines, no amine appeared in the Rf range 0.30-0.36. Although six are shown in this category in the normal subjects of Table I, they are absent from all but one of the normotensive samples in Table II. The frequent appearance in urine of hypertensive patients of an amine with an Rf above 0.60 and its absence from normal subjects (Tables I and II) agrees with the previous finding in hypertensive blood of an amine with an Rf of 0.62 which was not found in normotensive blood extracts.[12]

The total average number of daily urinary amines found by batch extraction in hypertensive and normotensive subjects is approximately 3.0. A somewhat higher average recovery (4.2) was found when the continuous extraction method was used. There is no clear evidence that the number of amines excreted by hypertensive persons is different from that for normal people.

TABLE III. PAPER CHROMATOGRAPHY OF PRIMARY AMINES IN URINE OF NORMAL SUBJECTS BY CONTINUOUS EXTRACTION USING BUTANOL-WATER SOLVENT*

SUBJECT	DAY	0.0 A	0.10 B	0.18 C	0.26 D	0.30 E	0.36 F	0.44 G	0.52 H	0.60 I	0.70
D. F. D.	1		x	x			x				
	2	x	x	x			x				
A. H. D.	1	x		x			x				
H. A. S.	1			x		x	x	x			
	2		x	x	x						
E. F. P.	1		x								
	2		x			x	x			x	
H. M. P.	1	x		x†			x	x			
	2	x†	x				x		x		

*Aliquot of extract applied to paper represented 5 per cent of each sample.
†Two spots measured in this Rf range.

Continuous Extraction.—Normal Subjects: Table III shows the distribution of Rf values of extracts representing 5 per cent of each of nine twenty-four-hour samples of urine from five normal subjects. An average of 3.2 amines were recovered. Little additional information was obtained by tripling the aliquot of extract applied. Recovery from 15 per cent of the total daily urine samples run on the same chromatograms did not increase the number of amines recovered in four of the nine urines and increased the average number detected per person by 0.8. Under these conditions the number which appeared per sample ranged from three to five. From the data there is a tendency for the same amines to appear on consecutive days in the same person but no clearly distinguishing characteristics in different persons. Six of the nine samples, after spraying, showed a large ninhydrin-reactive spot with an Rf in the hydrophilic range of 0.07. In several this was large enough to spread in all directions and distort other spots, but in some it was entirely absent. On exposure to room temperature for several days the spot faded rapidly to a pale yellow color. Because of its variable appearance even in the same individual it may be related to food intake. In E. F. P. on the first day this substance was practically absent, but on the following day it was spread between Rf 0.0 to 0.25.

Amine Profile in Patients With Miscellaneous Diagnoses.—A larger number of amines appeared after continuous extraction than was found by the batch method, in part because of the use of larger aliquots and in part because of more efficient extraction by the Wolf-Hirshberg apparatus. In each of seven twenty-four-hour urine samples collected from three patients with the endocrine hypertensive syndrome[13] and one patient with nephritis, an amine appeared with an Rf between 0.10 and 0.18 (Table IV). Neither of two patients with Laennec's cirrhosis had an amine with this Rf. Both of the cirrhotics, however, had an amine in the "c" range which occurred only once in the endocrine hypertensives and once in the nephritic. No distinguishing features were observed in a patient with anhidrosis.

TABLE IV. MISCELLANEOUS DIAGNOSES—DISTRIBUTION OF AMINES BY Rf (CONTINUOUS EXTRACTION)

PATIENT	DAY	HOURS EXTRACTED	NO. OF AMINES	Rf RANGES A 0.0	B 0.10	C 0.18	D 0.26	E 0.30	F 0.36	G 0.44	H 0.52	I 0.60	J 0.70
Endocrine Hypertensive Syndrome													
M. L.	1	10	8	x	x	x	–	x	x	x	x	x	–
	2	12	6	x	x	–	–	x	x	x	x	–	–
M. P.	1	12	3	–	x	–	x	–	x	–	x	x	–
L. Ro.	1	6	5	–	x	–	x	–	x	–	x	x	–
	2	6	4	–	x	–	–	–	x	–	–	x	x
Nephritis													
C. P.	1	16	6	x	x	x	–	x	x	–	x	–	–
	2	16	5	x	x	–	–	x	x	–	x	–	–
Cirrhosis													
C. N.	1	17	6	x	–	x	–	–	x	x	x	x	–
B. E.	1	12	7	x	–	x	x	x	x	–	x	x	–
Anhidrosis													
N. H.	1	12	7	x	x	x	x	–	x	x	–	x	–
	2	12	7	x	x	x	x	x	–	x	–	x	–

Effect of Mannitol Hexanitrate on Amine Excretion.—Continuous Extraction: Table V shows a comparison of the effect of placebo with that of Nitranitol on the amine profile in 11 patients. The data represents continuous extractions of 25 twenty-four-hour samples of urine. Twenty-four-hour specimens were extracted for a total of eight to sixteen hours in four- or eight-hour periods. The number of periods was disregarded in the totals given. In 6 of the 10 primary amine categories the amine was found in fewer daily samples while the patient was taking Nitranitol than while on placebo (a total of 35 compared with 19). In one Rf range an amine was found more frequently (five to four) after Nitranitol as compared with placebo. As stated previously the normal range is large and no attempt has been made to show this to be a significant difference.

Batch Extraction: These methods (Table VI) on some of the same samples failed to show a difference between the total number of amines excreted after placebo (32) and after Nitranitol ingestion (27). While six of the Rf ranges had a lower number of amines after Nitranitol (10) as compared with placebo

TABLE V. EFFECT OF MANNITOL HEXANITRATE (NITRANITOL) ON AMINE PROFILE OF URINE WITH CONTINUOUS EXTRACTION

PATIENT*	DOSE†	DAY	NO. OF EXTRACTIONS	FREQUENCY OF OCCURRENCE 0.0 A	0.10 B	0.18 C	0.26 D	0.30 E	0.36 F	0.44 G	0.52 H	0.60 I	0.70 J	
L. Ri.	P	1	1	1	–	1	–	–	1	–	–	–	–	
		2	2	2	2	–	2	1	–	2	1	–	–	
	N	1	1	1	1	–	1	–	–	–	1	–	–	
L. S.	P	1	2	–	–	2	–	–	–	2	–	–	–	
	N	1	2	–	–	2	–	–	–	2	–	–	–	
L. Ro.	P	1	2	–	1	1	–	–	–	1	–	2	1	
	N	1	2	–	1	–	1	–	2	–	2	2	–	
E. W.	P	1	1	1	1	–	–	–	–	–	–	–	–	
	N	1	1	–	–	–	–	–	1	1	–	–	–	
R. S.	P	1	2	–	2	2	1	–	1	–	2	–	–	
	N	1 & 2	2	1	2	2	1	–	1	–	2	–	–	
I. P.	P	1	1	–	–	1	–	–	–	1	1	–	–	
	N	1	1	1	–	1	–	–	1	1	–	–	–	
G. E.	P	1	2	1	2	1	–	–	2	–	1	–	–	
	N	1	1	1	–	–	–	–	–	–	–	–	–	
E. C.	P	1	3	3	1	1	–	–	2	–	–	–	–	
	N	1	3	3	2	1	–	–	–	1	–	–	–	
		2	2	2	–	–	–	–	–	–	–	–	–	
B. M.	P	1	4	4	1	1	1	1	1	–	1	–	–	
	N	1	2	2	2	–	2	–	–	–	2	–	–	
G. H.	P	1	3	3	3	1	–	–	–	–	–	–	–	
		2	2	2	1	1	–	1	1	–	–	–	1	
	N	1	2	2	1	1	–	–	–	–	–	–	–	
N. H.	P	1	2	2	2	1	1	1	–	2	–	1	–	
	N	1	2	2	2	2	1	–	1	2	1	–	–	
Total	P	13		9	10	11	4	4	6	5	5	2	2	58
	N	12		9	7	6	5	0	5	5	5	1	0	43

*All patients were hypertensive but N. H.
†P = Placebo. N = Nitranitol.

TABLE VI. EFFECT OF MANNITOL HEXANITRATE (NITRANITOL) ON BATCH EXTRACTION (1 EXTRACTION EACH DAY)

PATIENT	DOSE	DAY	0.0 A	0.10 B	0.18 C	0.26 D	0.30 E	0.36 F	0.44 G	0.52 H	0.60 I	0.70 J	
L. S.	P	1	–	1	–	1	–	1	1	–	1	–	
		2	1	–	1	–	–	–	–	–	1	–	
	N	1	–	–	1	–	–	–	–	1	–	–	
		2	–	–	1	–	–	–	1	1	1	–	
L. Ro.	P	1	–	–	1	–	–	–	1	–	–	1	
		2	1	–	1	–	–	–	–	–	–	–	
	N	1	–	–	1	–	–	–	1	–	–	1	
		2	1	1	–	–	–	–	–	1	–	–	
E. W.	P	1	1	–	1	–	–	–	1	–	–	–	
		2	1	1	–	–	–	1	1	–	–	–	
	N	1	–	1	–	–	–	–	1	1	–	–	
		2	1	1	–	–	–	1	1	–	–	–	
R. S.	P	1	–	1	1	–	–	–	–	–	–	–	
		2	1	1	1	–	–	–	–	–	–	–	
	N	1 & 2	1	1	–	–	–	–	–	–	–	–	
I. P.	P	1	–	1	1	–	–	–	–	–	–	1	
	N	1	1	–	–	–	–	–	1	–	–	–	
N. H.*	P	1	1	1	1	–	1	–	–	–	–	–	
	N	1	1	–	–	–	–	–	1	–	–	–	
		2	1	1	–	–	1	–	–	–	–	–	
Totals	P	10	6	6	8	1	1	2	4	0	2	2	32
	N	10	6	5	2	0	1	1	6	4	1	1	27

*Normotensive patient with anhidrosis.

(21), a similar comparison of random day-to-day variation shows (Table II) that five Rf categories showed an over-all change from 18 to 10 amines on two consecutive days while in three ranges there was an increase from 11 to 17 amines.

Chemical Characteristics of Naturally-Occurring Amines.—As indicated previously,[9] simple aliphatic amines can usually be distinguished from those with aromatic or heterocyclic substitutions by the ratio of the 405 to 570 mμ optical density readings of their ninhydrin complexes. In pure solution simple aliphatic compounds rarely give a ratio above 1.20 and substituted compounds usually have ratios above 1.30. In urinary extracts contamination of the chromatographed amines by other bases may modify these ratios slightly. When this occurs, the separation of the two groups becomes less distinct in that more aliphatic amines have ratios above 1.20. The ratios falling below 1.20 under these conditions, however, have so far been associated with aliphatic compounds. The known correlations between the number of carbon atoms in a simple aliphatic amine and its Rf value[9] helps further to characterize the urinary amines with low 405 to 570 mμ optical density ratios.

Table VII shows the distribution by Rf range of 62 urinary and 16 known aliphatic and ring-substituted amines from chromatograms run in a solvent of butanol saturated with water. Table VIII shows similar data for chromatograms run in a solvent made up of water, butanol, and isopropanol in the proportions 2:3:5. The data in no way identify the unknowns, but offer a lead toward their final identification. Unknowns with 405 to 570 mμ optical density ratios between 1.20 and 1.30 are considered indeterminate and are not included in either the aliphatic or ring-substituted groups. Oxygen, primary amine, and sulfur substitutions lower Rf values; only cyclic substitutions are known to raise it. Therefore, the chemical nature of amines with low ratios and high Rf's is more sharply limited to the unsubstituted straight or branched aliphatic compounds.[9] An exception is seen in β-phenylethylamine which has a ratio in the aliphatic range. That of α-phenylethylamine is higher but still unusually low. The naturally-occurring compounds with low Rf's and low ratios may be diamines or poly-hydroxy bases such as glucosamine. A large number of primary amines which are practically stationary in butanol saturated with water are recorded as ring-substituted compounds, but at this Rf spuriously high ratios may occur because of the presence of colored bases which do not react with ninhydrin and do not move on chromatograms.

Estimate of Daily Output of Amines.—In addition to the variability in the number of primary amines excreted, there is obviously a wide range in their quantity from day to day. Since none of them has been identified, their distribution and extinction coefficients are not known.

From the recovery experiment previously described, it is estimated that at least 50 per cent of aliphatic amines and larger amounts of the ring-substituted compounds are lost in extraction and chromatography under these conditions. When Tyramine was added to urine which had been cleared previously by extraction for twenty-four hours, only 11.3 per cent was recovered

TABLE VII. DISTRIBUTION BY Rf RANGE OF ALIPHATIC AND RING-SUBSTITUTED PRIMARY AMINES FROM URINE AFTER PAPER CHROMATOGRAPHY IN BUTANOL-SATURATED WATER

ALIPHATIC					RING-SUBSTITUTED			
Rf RANGE	KNOWN AMINE	MEAN 405/570 mμ RATIO	MEAN Rf	NO. OF URINARY AMINES*	KNOWN AMINE	MEAN 405/570 mμ RATIO	MEAN Rf	NO. OF URINARY AMINES*
				405/570<1.20				405/570>1.30
0.00-0.10	Glutamine		0.025	11	Histamine	1.79	0.082	14
	Methyl-amine	1.18	0.10					
			0.11	4				
0.10-0.18	Cystamine				Norepi-nephrine	1.57	0.13	9
	Ethyl-amine	1.10	0.17					
0.18-0.26				3	Serotonin		0.20	5
0.26-0.30	n-Propyl-amine	1.23	0.28	1				
0.30-0.36					Tyramine	1.24	0.33	2
0.36-0.44	n-Butyl-amine	1.06	0.44	3	Tryptamine	1.33	0.38	3
0.44-0.52				1	ß-Phenyl-ethylamine	1.09	0.48	1
					α-Phenyl-ethylamine	1.12	0.53	2
0.52-0.60	n-Amyl-amine	1.09	0.53					
	Isoamyl-amine	0.99	0.53	1				
0.60-0.70	n-Hexyl-amine	0.99	0.66					1

*Number of amines in each Rf range giving sufficient color to read spectrophotometrically.

TABLE VIII. DISTRIBUTION BY Rf RANGE OF ALIPHATIC AND RING-SUBSTITUTED PRIMARY AMINES FROM URINE AFTER PAPER CHROMATOGRAPHY IN WATER-BUTANOL-ISOPROPANOL MIXTURE*

ALIPHATIC					RING-SUBSTITUTED			
Rf RANGE	KNOWN AMINES	MEAN 405/570 mμ RATIO	MEAN Rf	NO. OF URINARY AMINES†	KNOWN AMINE	MEAN 405/570 mμ RATIO	MEAN Rf	NO. OF URINARY AMINES†
				405/570 <1.20				405/570 >1.30
0.00-0.10	Glutamine	1.18	0.098	6				1
0.10-0.18	Glucosamine	1.13	0.11	2	Histamine	1.73	0.16	2
0.18-0.26								1
0.26-0.30	Ethanol-amine	1.16	0.27		Norepi-nephrine	1.10	0.30	2
	Methyl-amine	1.17	0.29					
0.30-0.36				4				6
0.36-0.44	Ethylamine	1.05	0.37	7				5
0.44-0.52	n-Propyl-amine	1.10	0.44		Tyramine	1.37	0.48	1
					Trypt-amine	1.24	0.51	
0.52-0.60	n-Butyl-amine	1.11	0.53	1				1
0.60-0.70	n-Amyl-amine	1.01	0.63					
	Isoamyl-amine	1.09	0.63	3	β-Phenyl-ethyl-amine	0.83	0.62	4
0.70 +	n-Hexyl-amine	1.04	0.71					
	Decylamine	1.10	0.76	2				

*Water, butanol, and isopropanol in proportions 2:3:5.
†Number of amines in each Rf range giving sufficient color to read spectrophotometrically.

in twelve hours of extraction. The low recovery was probably a result of both destruction of the Tyramine and its distribution coefficient, for the carbon tetrachloride became yellow after eight hours of extraction. Norepinephrine and other catechols are oxidized by strong alkali and were not tested.

As little as 2 μg of most amines of biologic interest can be detected on paper chromatograms. Assuming 50 per cent recovery by continuous extraction of four-fifths of a twenty-four-hour sample, by this method the minimum amount of amine still detectable is approximately 5 μg. Usually smaller aliquots are used and most of the amines represented in the tables were present in concentrations of at least 20 μg per day. By comparison of optical density readings of unknown with known amines eluted from chromatograms, it is estimated that the excretion rate of any amine rarely, if ever, exceeds 500 μg and, in general, does not exceed 100 μg per day.

DISCUSSION

The complete metabolic pathways have not been elucidated for any of the amino acids in the diet of man. The major pathways of several have been followed, but in no case has 100 per cent of the nitrogen been accounted for. The possibility that a variable fraction of the amino acids may normally be decarboxylated without deamination has been neither demonstrated nor disproved. The presence of up to eight primary amines in urine of normal subjects and hypertensive and other patients is strongly suggestive that such a reaction occurs. Gurin and Delluva have presented chemical evidence that such decarboxylation occurs in the conversion of phenylalanine to epinephrine,[14] and Stetten has demonstrated conversion of glycine[15] and serine[4] to ethanolamine. Physiologic evidence of decarboxylation has been obtained by Holtz,[16, 17] and by Bing and Zucker.[18] Alternatively, the amines could be formed by transamination reactions.

The small amounts of amines present in urine are in accord with the known facts about the intermediate metabolism of amino acids. For example, it was found that 83 to 92 per cent of the isotopic carbon in glycine-2-C^{14} administered intravenously to patients was recovered as $C^{14}O_2$ in the expired air.[19] Only 5 per cent was excreted by the kidneys. Part of this may have been excreted as ethanolamine or methylamine. Normally, the amino acids in the urine are present in amounts averaging from 5 to 25 mg. per twenty-four hours,[20] which means that the amines are excreted in amounts of roughly 1 to 5 per cent of that of the amino acids.

Up to the present time, histamine and norepinephrine are the only two primary amines demonstrated to have an etiologic role in the production of diseases. The relation of other amines to signs or symptoms of disease is not known, although most are vasoactive agents. Several of us in this laboratory have experienced the subjective effects of isoamylamine injected intravenously. It produces flushing and warmth of the skin, particularly of the face, but in the extremities as well. The apprehension experienced was consistent enough to be of pharmacologic rather than psychogenic origin. The possibility that

the tone and quality of an emotional state, as well as such symptoms as headache and anginal pain, may be governed by release of such compounds deserves study.

It has been reported[21, 22] that the organic nitrates bring about relief of symptoms associated with hypertension, particularly those of anginal pain, and may either cause or relieve headaches. If such symptoms are brought on by specific amines, it would be interesting to know whether the amine profile would be changed by an organic nitrate. Action of an amine could be blocked by the drug, thus increasing its rate of excretion. On the other hand, the amine could be destroyed or combined, resulting in an apparent decrease in the quantity excreted. The first approach to this problem has been presented here. Much more work is necessary before the full significance of the amines and their relationship to different forms of therapy is known.

The results of qualitative analysis and of pharmacologic assay of these substances for vasoactivity will be reported in subsequent papers.

CONCLUSIONS AND SUMMARY

Primary amines falling into 10 Rf bands have been separated from urine of normal subjects and of hypertensive and other patients. Single twenty-four-hour urine samples have contained as many as eight amine spots on paper chromatograms. The individual and daily variability in the number of amines excreted is considerable. The primary amines are usually excreted in an order of magnitude of 20 to 100 μg per day, approximately 1 to 5 per cent of the amount of amino acids excreted daily. The ingestion of mannitol hexanitrate (Nitranitol) on the primary amine profile in urine was studied. Differences which were observed could have been due to random variability. An effect on single primary amines cannot be ruled out.

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COCCIDIOIDOMYCOSIS—POSSIBLE FOMITE TRANSMISSION

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THE disease coccidioidomycosis was first reported from South America and was subsequently found to occur in California as an endemic disease spread by the spores of the fungus *Coccidioides immitis*. The spores are found on plants and in soil and are naturally spread to man in dust. The gradual widening of the known endemic areas is a familiar story and it is sufficient to say that at present known endemic areas in the United States include California, Arizona, New Mexico, Texas, and probably bordering states.[4, 7, 9]

Despite the fact that *Coccidioides immitis* produces hardy desiccant and heat-resistant forms, and the disease is readily contracted by brief contact with viable organisms,[10-12] no fomite transmission of this disease has been reported. No naturally contracted cases of coccidioidomycosis have been reported from the states of Illinois, Indiana, Ohio, Pennsylvania, New York, or the New England States. Reports of sporadic cases found in these areas but originating elsewhere occur.[3, 5, 9, 13]

Laboratory transmission of the fungus has been reported on several occasions. This apparently results from the spread of spores from cultures. Reversion of skin tests to positive occurred in one laboratory prior to the establishment of a special safety cabinet for working with cultures.[14] Dr. Charles E. Smith suggests that "the environment of a laboratory in which Coccidioides is handled is comparable to that of a coccidioidal endemic area."[14]

It is our purpose to present the case report of a patient who lived and traveled only in nonendemic areas of coccidioidomycosis infection and who did not work in a laboratory. We wish to present the suggestion of fomite transmission so that others may keep watch for this phenomenon.

CASE HISTORY

This 30-year-old Negro man was born June 22, 1921, in Boston, Massachusetts. He remained in Boston until the age of three years, at which time his family moved to Chicago, Illinois, where he has since resided. He denies ever taking any trips outside of the city. There were no known childhood illnesses.

At the age of 19 years, he started working for a spring corporation in Chicago, Illinois. The work consisted of covering springs with a product called "sisal." This is a hemp product imported from dry areas of California and Mexico. The process involved the production of considerable dust, but caused no symptoms. He performed this task for two years and was then drafted into the Army.

The patient served in the Army from Jan. 25, 1943, to Feb. 7, 1946. His entire Army service was spent at two posts: Camp Grant, Illinois, and Camp Miles Standish, Massachusetts.

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His only traveling consisted of trips between these two camps and Chicago, Illinois. Duties were those of a trainee and, finally, supply sergeant. He never served as a medical technician.

The patient had a single isolated episode of fever and malaise in 1943, for which he was hospitalized at the Marine Hospital, Chicago, Illinois. This subsided spontaneously and the patient's illness was diagnosed as acute catarrh. He remained well throughout the remainder of his military service.

After discharge from military service he returned to work at the spring corporation in Chicago, this constituting his latest contact with the material "sisal." During this period of February, 1946, to March, 1947, he had several severe attacks of upper thoracic pain necessitating physician's care, but no chest x-ray or diagnosis was accomplished.

The patient remained apparently well until May, 1949, at which time he developed a chronic cough, but the chest x-ray is stated to have been normal.

In June, 1949, the patient developed a mass in the left lumbar area accompanied by pain and elevation of temperature. He was hospitalized at a large charity hospital and treated for six months for tuberculosis. Toward the end of his stay the organism was identified from aspirated material as that of *Coccidioides immitis*. During this period he had lost 20 pounds. The patient was transferred to the Veterans Administration Hospital, Hines, Illinois.

He received potassium iodide, and deep x-ray therapy to the cervical, dorsal, and lumbar spines with rather marked improvement. Pertinent findings on admission were limited to evidence of weight loss and findings in the spine. Slight fullness was present in the left flank, and definite limitation of motion was present throughout the back, and local tenderness was noted to the left of the second and third lumbar vertebrae. X-ray revealed erosions of the body of the fourth, fifth, and sixth cervical, the eleventh and twelfth dorsal, and third, fourth, and fifth lumbar vertebrae. There was a large paravertebral abscess extending from the sixth to the twelfth dorsal vertebrae. Aspirations from chest and lumbar areas over the next two years yielded 18 consecutively positive cultures for *Coccidioides immitis*. The material showed "hyphae formation with specialized side branches showing formation of chlamydospores." The typical spores of *Coccidioides immitis* were seen. These cultures showed grayish-white colonies growing that were cottony in appearance. After the culture stood for a time, the color changed to brownish.[11] (One per cent gentian violet and Azochloramid were instilled into these abscesses with no apparent help in diminution of the material formed.) Blood chemistry studies revealed only a persistently reversed albumin-globulin ratio. Repeated sputa were negative on culture for *Coccidioides immitis*. No appreciable change occurred over eleven months in the spine x-rays. On Feb. 1, 1950, a coccidioidal complement fixation was performed by the School of Public Health at the University of California* with the following results:

Serial dilutions of serum (0.25 c.c.)							
1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
						0	0

Conclusions were "definite confirmation of diagnosis of disseminated coccidioidal infection (coccidioidal granuloma)."

This patient has been seen at monthly intervals since last discharged on Sept. 19, 1951. He continues to slowly reform fluid in the lumbar abscess, but at a decreasing rate. No pulmonary infiltration has ever developed and the spine x-rays remain stable.

A small sample of "sisal" was obtained and washings cultured; these failed to grow *Coccidioides immitis*.

DISCUSSION

As previously stated, the well-established endemic area for coccidioidomycosis lies within the states of California, Arizona, New Mexico, Texas,[4, 9] and areas in South America.[2] Sporadic cases have been reported from bordering areas of Colorado, Kansas, Washington, and Mexico.[13] In addition to the above areas

*We are indebted to Dr. Charles E. Smith for the performance and interpretation of the complement fixation tests.

in the United States, there are reports of cases occurring in the South[7] and Midwest.[1] These cases will be discussed in more detail. The case of Hirsch reported in a Negro man in Chicago will not be discussed as the patient was a Pullman porter traveling through the endemic areas and presumably contracted the disease there.[3] Aside from these instances, no case has been reported outside the endemic area, although laboratory infections have occurred.[5]

In 1920 Lynch[7] reported a case diagnosed on the basis of postmortem examination without cultural proof. This woman lived on an island off the Carolina coast and had not, as far as known, been out of the state. A sister had a disease described as "similar." History was unfortunately unreliable so that true areas of travel were not obtainable. Confusion with other mycotic infections was possible and, unfortunately, neither cultural nor serologic proof was available. In our opinion this interesting case must remain of uncertain value from an epidemiologic sense.

In 1933 Beaver and Furrer[1] reported a case with the microscopic appearance of coccicioidomycosis in a 41-year-old man who had been born in Germany and immigrated to Iowa, then to Minnesota. No history of travel elsewhere was given. No cultures were possible due to previous embalming prior to autopsy. No serologic testing was, of course, possible. The authors felt as we do that this is not a proved, but rather a possible, case of coccidioidal granuloma.

Montessori, in 1947,[8] reported four cases of mycotic infection with organisms resembling *Coccidioides immitis* (1 Cryptococcus, 1 *Glenospora neutropaea*, and 2 *Coccidioides immitis var. meteruopaeus*). All of these individuals worked for a grain importing company which received its imports from America. This is the first and only group to date implying possible fomite transmission.

Loosli and associates[6] demonstrated increased coccidioidin sensitivities in students from endemic areas and also appreciable sensitivity among students from Illinois, Indiana, and Ohio. They claim considerable evidence for cross reaction between the antigens of coccidioidomycosis and histoplasmosis. No such cross sensitivity was found in our patient.

This patient represents a case of coccidioidomycosis of unknown origin. Either contraction within a previously not known endemic area or contraction by fomite transmission seems the likely form of contagion. Our attempt to demonstrate fomite transmission via "sisal" failed, as might have been expected. Only chance alone might have given us a positive specimen years after the purported contagion. Specimens obtained at different times of the year and from different areas with different modes of transport render this method unreliable. An additional attempt to get at the problem was carried out by attempting to contact and skin test co-workers of the patient. This unfortunately failed due to lack of cooperation and the length of time elapsed since possible contagion. A comparison of these tests with those figures of Loosli might have been illuminating.

The absence of known serious disease among co-workers of the patient does not invalidate the idea of fomite transmission, as in endemic areas the percentage of acute cases going on into the chronic form is fortunately very small.

That this patient might have contracted his disease within previously not recognized endemic areas is possible but seems unlikely, as does person-to-person transmission. As previously noted, no definitely proved cases have ever come from these areas without having contact with endemic areas and no person-to-person transmission has been proved to our knowledge. We, therefore, wish to add this case to those of Montessori as a likely case of fomite contraction. Further diligent search for such cases is recommended so that evaluation of potential industrial hazards can be carried out.

SUMMARY

A proved case of coccidioidomycosis due to *Coccidioides immitis* is presented in a patient never in a known endemic area. Daily contact with dusty materials imported from endemic areas brings up the problem of fomite transmission.

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THE STABILITY OF HUMAN COMPLEMENT-FIXING ANTIBODIES AGAINST LYMPHOGRANULOMA VENEREUM, PSITTACOSIS, INFLUENZA A, AND INFLUENZA B ANTIGENS UNDER VARIOUS CONDITIONS OF STORAGE

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WITH the increasing use of the complement fixation technique as a routine procedure for the diagnosis of viral and rickettsial diseases, it has become extremely important to determine the stability of the antibodies under the various conditions of storage to which sera may be subjected. Few investigations have been made on this problem. Howitt[1] has stated that complement-fixing antibodies for lymphocytic choriomeningitis in immune guinea pig serum could be demonstrated in sera which had been stored at icebox temperature for one year and ten months. Enders and co-workers[2] have stated that neither human nor monkey mumps immune sera displayed a loss in complement-fixing titer after storage for six months in a carbon dioxide cabinet. However, at a temperature of 4° C., the level of complement-fixing antibodies for mumps in human serum was said to decrease rapidly, with no demonstrable antibodies remaining after eight months unless the initial titer was very high. It has been recommended by Casals[3] that human sera which are to be tested for the presence of complement-fixing antibodies against viral and rickettsial encephalitides be kept at 4° C. until the test was performed, and if the sera could not be tested within a few days after being drawn, they should be frozen and kept in the dry icebox. In a recent study Bennett and Kempf[4] have reported that human mumps convalescent sera in flame-sealed, chemically clean, glass ampules showed no loss in complement-fixing antibody titer after two months' storage at -15° C. or after one week's storage at temperatures of 5° C., 22° to 30° C., or 37° C.

The purpose of the following experiments was to determine what methods of storage would prove to be the most satisfactory for storing human sera containing complement-fixing antibodies against lymphogranuloma venereum, psittacosis, influenza A, and influenza B antigens.

Three different temperatures of storage were studied. The stability of these complement-fixing antibodies at room temperature (21° to 30° C.) was studied in order to determine whether or not sera might be shipped to diagnostic laboratories without refrigeration. It also seemed advisable to investi-

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gate the stability of complement-fixing antibodies at icebox temperature—the temperature at which sera are usually maintained prior to being tested for complement-fixing activity. Because freezing sera in the carbon dioxide freezer is a relatively new method for preserving immune sera, the effects of these temperatures upon the antibody titers of human sera were investigated. Also, the effects upon antibody titers of repeated freezing at freezer temperatures and thawing at 57° C. were studied.

In addition, the stability of complement-fixing antibodies in human sera stored in both screw-capped tubes and flame-sealed ampules was investigated.

MATERIALS AND METHODS*

Fresh, pooled guinea pig serum was used for complement, and two full units in a volume of 0.2 ml. were used in all of the tests.

Sensitized sheep cells were prepared by mixing equal amounts of a dilution of antisheep hemolysin containing two units in a volume of 0.2 ml. and 2 per cent washed sheep erythrocytes. In the tests the sensitized cells were added in a volume of 0.4 ml.

Blood was freshly drawn from persons whose sera previously had been shown to contain antibodies against one of the four antigens mentioned previously. Twenty to thirty milliliters of blood were withdrawn by venipuncture and allowed to clot at room temperature, and cell-free serum prepared by centrifugation. On the same day on which the specimen was obtained, a portion of the serum was inactivated in a water bath at 57° C. for thirty minutes and twofold dilutions of the serum were made in physiologic saline and tested against the complement-fixing antigen against which it had previously been shown to contain antibodies. Parallel dilutions of the serum were also tested against antigen controls prepared from normal tissues. In this manner, the original titer of the serum before storage was obtained. Sera which were stored in screw-capped tubes were dispensed into three of the tubes, each of which was stored at a different temperature—one at room temperature (21° to 30° C.), one at icebox temperature (4° to 10° C.), and one at carbon dioxide freezer temperature (-50° to -70° C.). At weekly intervals extending over a period of six weeks, aliquots of serum were removed, inactivated at 57° C. for thirty minutes and the three portions of serum were run in parallel against their homologous antigens. Sera which were stored in flame-sealed glass ampules were dispensed into 18 ampules which were then flame-sealed. Six of these were stored at room temperature, six at icebox temperature, and six in the carbon dioxide freezer. Each week an ampule which had been stored at each of the three temperatures was opened, the serum was inactivated, and the three portions were tested in parallel against their homologous antigens. In this way, comparisons could be made of the effects of the three temperatures of storage and the two types of storage tubes upon the original antibody titers of the sera.

The procedure for the complement fixation tests was as follows: Serial twofold dilutions of the serum to be tested were added to a series of tubes in 0.2 ml amounts. To each dilution of serum 0.2 ml. of complement (two units) and the specified amount and dilution of antigen were added. These reagents were incubated at 37° C. for eighty minutes, after which 0.4 ml. of sensitized sheep cells was added to each tube and incubation was continued for an additional thirty minutes at 37° C. The antisheep hemolysin and 2 per cent erythrocytes were mixed together thirty minutes prior to their addition to the test system. Results were reported in terms of 4+ for no hemolysis, 3+ for 25 cent hemol-

*The complement-fixing antigens used in these studies were obtained from Markham Laboratories of Chicago, Illinois; Lederle Laboratories of Pearl River, New York; Parke, Davis & Company, Detroit 32, Michigan; E. R. Squibb & Sons, New Brunswick, New Jersey; and The Director of Sectional Research in Microbiology of The National Institutes of Health. Antigen controls made from normal tissues, as well as positive animal antisera for controls were obtained from Markham Laboratories, and The Director of Sectional Research in Microbiology of The National Institutes of Health. The dilutions and volumes of antigens used in the tests were those recommended by the manufacturers of each antigen.

ysis, 2+ for 50 per cent hemolysis, and 1+ for 75 per cent hemolysis. The results were checked against a "reading scale" as suggested by Kolmer and associates.[5] The titer of the serum was determined as the highest dilution which gave a 4+ or a 3+ reaction.

In checking the reproducibility of our method, it was found that in 120 instances in which complement fixation tests were repeated on a serum on the same day or at least within one week after storage in the icebox, that exactly the same titers were obtained upon repetition in 109 or 90.8 per cent of the cases. In the balance of these tests, variations of plus or minus twofold occurred in 10 instances and fourfold in one instance. Although there are occasional discrepancies in complement-fixing antigens, if antigens are carefully checked against known positive sera, accurate results can usually be obtained in complement fixation tests for viral and rickettsial diseases. In these studies, an additional check upon the accuracy of the tests was furnished by running the three portions of serum in parallel against the same antigen dilution on the same day.

RESULTS

A. *Stability of Complement-Fixing Antibodies in Human Sera Stored in Screw-Capped Tubes.*—

1. *At room temperature:* Table I shows the degree to which the original antibody titers of 38 human sera dispensed in screw-capped tubes persisted over a period of six weeks at room temperature. The number of sera which retained their original titers at each time interval is indicated.

The original complement-fixing titers of these sera ranged from 1:8 to 1:256. The results presented in Table I show a decrease in complement-fixing titers of these sera over the six-week period of time. It should be noted, however, that a significant number of sera, as determined by the chi-square procedure, retained their original titers after three weeks of storage at room temperature. A significant number of decreases in antibody titer occurred between three and four weeks in sera containing antibodies against psittacosis, and between four and six weeks in sera containing antibodies against lymphogranuloma venereum, influenza A, and influenza B. The decreases in antibody titer which occurred in these sera over the six-week period were slight. They were decreases of twofold or fourfold; and in no instance did the complement-fixing activity disappear completely from the sera.

2. *At icebox temperature:* Table II gives the number of sera stored in screw-capped tubes which retained their original titers after each period of

TABLE I. THE LENGTH OF TIME SERA STORED IN SCREW-CAPPED TUBES RETAINED THEIR ORIGINAL COMPLEMENT-FIXING ANTIBODY TITERS WHEN STORED AT ROOM TEMPERATURE

TYPE OF ANTIBODIES CONTAINED IN SERA	NUMBER OF SERA	NUMBER OF SERA WHICH RETAINED ORIGINAL ANTIBODY TITERS AFTER TIME INTERVALS OF: ONE WEEK	TWO WEEKS	THREE WEEKS	FOUR WEEKS	SIX WEEKS
Lymphogranuloma venereum	7	7	7	6 ($X^2 = .08$)	3 ($X^2 = 3.1$)	1 ($X^2 = 7.3$)
Psittacosis	8	8	8	6 ($X^2 = .57$)	3 ($X^2 = 4.6$)	1 ($X^2 = 9.2$)
Influenza A	8	8	8	7 ($X^2 = .06$)	6 ($X^2 = .57$)	0 ($X^2 = 12.2$)
Influenza B	15	15	15	15	11 ($X^2 = 2.7$)	1 ($X^2 = 22.6$)
Total	38	38	38	38	23	3

Probability of a chi-square value larger than 3.841 using one degree of freedom = 0.05.

storage at icebox temperature. At this temperature in the sera containing each specific type of antibody, a significant number of decreases in titer did not occur until after four weeks. Here again, the decreases in antibody titer during this six-week period were slight and were only decreases of twofold or fourfold.

3. *At carbon dioxide freezer temperature:* Table III gives the number of sera stored in screw-capped tubes which maintained their original antibody titers against the four antigens at various intervals during six weeks of storage in the carbon dioxide freezer.

TABLE II. THE LENGTH OF TIME SERA STORED IN SCREW-CAPPED TUBES RETAINED THEIR ORIGINAL COMPLEMENT-FIXING ANTIBODY TITERS WHEN STORED AT ICEBOX TEMPERATURE

TYPE OF ANTIBODIES CONTAINED IN SERA	NUMBER OF SERA	NUMBER OF SERA WHICH RETAINED ORIGINAL ANTIBODY TITERS AFTER TIME INTERVALS OF: ONE WEEK	TWO WEEKS	THREE WEEKS	FOUR WEEKS	SIX WEEKS
Lymphogranuloma venereum	7	7	7	7	6 ($X^2 = .08$)	3 ($X^2 = 3.2$)
Psittacosis	7	7	7	7	5 ($X^2 = .58$)	2 ($X^2 = 4.9$)
Influenza A	8	8	8	8	6 ($X^2 = .57$)	4 ($X^2 = 3.0$)
Influenza B	15	15	15	15	11 ($X^2 = 2.6$)	4 ($X^2 = 4.2$)
Total	37	37	37	37	28	13

Probability of a chi-square value larger than 3.841 using one degree of freedom = 0.05.

TABLE III. THE LENGTH OF TIME SERA STORED IN SCREW-CAPPED TUBES RETAINED THEIR ORIGINAL COMPLEMENT-FIXING ANTIBODY TITERS WHEN STORED IN THE CARBON DIOXIDE FREEZER

TYPE OF ANTIBODIES CONTAINED IN SERA	NUMBER OF SERA	NUMBER OF SERA WHICH RETAINED ORIGINAL ANTIBODY TITERS AFTER TIME INTERVALS OF: ONE WEEK	TWO WEEKS	THREE WEEKS	FOUR WEEKS	SIX WEEKS
Lymphogranuloma venereum	8	4 ($X^2 = 3.0$)	3 ($X^2 = 4.6$)	2 ($X^2 = 6.3$)	1 ($X^2 = 9.0$)	0 ($X^2 = 12.3$)
Psittacosis	8	4 ($X^2 = 3.0$)	2 ($X^2 = 6.3$)	2 ($X^2 = 6.3$)	0 ($X^2 = 12.3$)	0 ($X^2 = 12.3$)
Influenza A	10	5 ($X^2 = 3.3$)	1 ($X^2 = 12.9$)	0 ($X^2 = 16.2$)	0 ($X^2 = 16.2$)	0 ($X^2 = 16.2$)
Influenza B	15	8 ($X^2 = 6.7$)	7 ($X^2 = 8.5$)	6 ($X^2 = 10.0$)	4 ($X^2 = 14.3$)	2 ($X^2 = 19.6$)
Total	41	21	13	10	5	2

Probability of a chi-square value larger than 3.841 using one degree of freedom = 0.05.

A striking difference will be noted between the number of sera maintaining their original titers at icebox temperature and the number maintaining their original titers at freezer temperature at each time interval. At icebox temperature sera in screw-capped tubes retained their original titers during three weeks of storage, while 50 per cent of the sera in screw-capped tubes stored in the freezer showed a drop in titer after just one week. Statistical analysis by the chi-square method shows that the number of sera containing influenza B antibodies showing a drop in titer was significant at one week, and the number of sera containing lymphogranuloma venereum antibodies,

psittacosis antibodies, and influenza A antibodies showing a drop in titer was significant at two weeks. During the six-week period of storage in the freezer most of the decreases in titer were of twofold or fourfold, but 20 per per cent of the anti-influenza A and 13 per cent of the anti-influenza B sera displayed decreases in titer of sixfold. Nine and a half per cent of the anti-influenza A and 4 per cent of the anti-influenza B sera showed decreases in titer of as much as eightfold. At room temperature and icebox temperature, none of the decreases in titer had been greater than fourfold.

Another very interesting fact observed was that the storage of antisera in screw-capped tubes in the freezer caused some of the sera to manifest a prozone reaction in complement fixation tests. Low dilutions of some of the sera stored in the freezer often failed to fix complement with homologous antigens, whereas higher dilutions of the sera gave positive complement fixation. While some freshly drawn sera have been observed to give a prozone reaction, there was, however, a definite correlation noted between conditions under which sera were stored and the degree to which prozones were manifest. This is illustrated in Table IV, which shows that in antilymphogranuloma venereum sera prozone reactions were most extensive after storage in screw-capped tubes in a carbon dioxide freezer.

TABLE IV. THE RELATIONSHIP OF THE METHOD OF STORAGE TO THE MANIFESTATION OF PROZONES IN SERA CONTAINING COMPLEMENT-FIXING ANTIBODIES AGAINST LYMPHOGRANULOMA VENEREUM ANTIGENS

SERUM NUMBER	HIGHEST COMPLEMENT-FIXING TITER OF SERUM	DILUTIONS OF SERUM STORED IN SCREW-CAPPED TUBES IN WHICH PROZONES OCCURRED AFTER THE FOLLOWING TEMPERATURES OF STORAGE:		
		ROOM TEMP. (21°-30° C.)	ICEBOX TEMP. (4°-10° C.)	FREEZER TEMP. (-50° C. AND LOWER)
1	1:128	No prozone	1:2, 1:4, 1:8	1:2, 1:4, 1:8
2	1:256	No prozone	No prozone	1:2
3	1:16	1:2	1:2	1:2, 1:4
4	1:32	No prozone	No prozone	1:2, 1:4, 1:8, 1:16
5	1:32	1:2	1:2	1:2, 1:4, 1:8, 1:16
6	1:128	No prozone	No prozone	1:2, 1:4
7	1:32	No prozone	No prozone	1:2, 1:4
8	1:16	No prozone	No prozone	1:2, 1:4, 1:8
9	1:32	No prozone	1:2	1:2
10	1:32	No prozone	No prozone	1:2

Although the manifestation of prozones after storage of sera in the carbon dioxide freezer was most frequently observed in sera containing antibodies for lymphogranuloma venereum or psittacosis, less extensive prozones were also observed in portions of some anti-influenza A and B sera which were stored in screw-capped tubes in the carbon dioxide freezer. It should be stressed that these prozones could be demonstrated repeatedly on the same specimen.

The results of our studies upon the stability of complement-fixing antibodies in sera in screw-capped tubes stored at three different temperatures are summarized in Table V. This table gives the number of parallel tests on portions of the same sera stored at the three temperatures in which the lowest

antibody titers occurred after storage at each of the three given temperatures. The sera represented in this table include those previously described on which the antibody titers were determined at weekly intervals for six weeks, and additional sera on which fewer parallel tests were run. The length of time these sera were stored before being run in parallel tests ranged from one week to six weeks.

TABLE V. THE NUMBER OF SERA IN WHICH THE LOWEST COMPLEMENT-FIXING ANTIBODY TITERS OCCURRED AFTER STORAGE IN SCREW-CAPPED TUBES AT THREE GIVEN TEMPERATURES

TYPE OF ANTIBODIES CONTAINED IN THE SERA	NUMBER OF SERA	NUMBER OF SERA IN WHICH THE LOWEST TITER OCCURRED AFTER STORAGE AT:		
		ROOM TEMP.	ICEBOX TEMP.	FREEZER TEMP.
Lymphogranuloma venereum	21	1	2	18
Psittacosis	22	2	1	19
Influenza A	26	2	0	24
Influenza B	45	4	1	40
Total	114	9	4	101

Lymphogranuloma venereum chi-square	−8.88
Psittacosis chi-square	−9.68
Influenza A chi-square	−19.02
Influenza B chi-square	−26.23
Degrees of freedom = 2	

Analysis of the data by the method of chi-square shows that in the case of lymphogranuloma venereum antisera the probability that such a variation occurred due to chance alone is between 0.02 and 0.01. In the case of the other three antibody-containing sera, the probability that the variation occurred due to chance alone is below 0.01. Therefore, the greater number of all four types of sera showing the lowest titers after freezer storage may be considered significant.

4. *Effects of repeated freezing and thawing upon complement-fixing antibody titers of human sera stored in screw-capped tubes:* The possibility was taken into consideration that the decreases in titers of portions of serum in screw-capped tubes stored in the freezer might be brought about by the process of freezing the serum and thawing it before each test was run. In order to determine if this were the case, portions of the sera stored in screw-capped tubes were subjected to repeated freezing at freezer temperatures and rapid thawing at 57° C. This process was carried out fifteen times on each portion of serum. These portions were then run in parallel with aliquots of the same serum which had been frozen once and left in the freezer. The total length of time both portions of serum remained in the frozen state was identical. No significant differences could be detected in the titers of these two portions of serum, which indicated that the decrease in titer was not brought about by repeated freezing and thawing.

5. *Effect of filtering upon complement-fixing antibody titers of human sera stored in screw-capped tubes:* Another possible explanation for the lower titers of sera in screw-capped tubes which had been stored in the freezer was that the freezing process had caused the precipitation of serum lipids or other

serum constituents which might interfere with the combination of antigen and antibody or with the combination of this complex with complement, thereby causing the serum to exhibit a lower titer. To test this possibility, sera which had been frozen in the freezer were thawed and divided into two parts, one of which was filtered through Whatman No. 42 filter paper before it was inactivated and run in parallel with the unfiltered portion. The titers of the filtered portions were not significantly different from those of the unfiltered portions. Therefore, it must be concluded that the lower titers are not due to interference by substances such as precipitated lipids which can be removed by filtering the serum through Whatman No. 42 filter paper.

The next aim of our investigations was to determine whether it was actually the low temperatures obtained in the carbon dioxide freezer which caused a drop in antibody titer of sera stored in screw-capped tubes, or if the drop in titer might be due to the absorption of carbon dioxide by the serum and the resulting drop in pH.

B. *Changes in pH of Human Sera Stored in Screw-Capped in the Carbon Dioxide Freezer.*—In order to determine if sera stored in screw-capped tubes in the carbon dioxide freezer might actually absorb enough carbon dioxide to lower their pH significantly, the pH of sera stored under these conditions was checked weekly for a period of six weeks; these pH values were compared with the pH values obtained on portions of the same sera stored in screw-capped tubes in the refrigerator. It was found that the pH of sera stored in screw-capped tubes in the carbon dioxide freezer might drop to as low as 6.0 in two weeks' time, whereas the pH of sera stored in screw-capped tubes in the icebox increased slightly—usually 0.3 to 0.4 pH unit in two weeks' time.

C. *The Stability of Complement-Fixing Antibodies in Human Sera Stored in Flame-Sealed Glass Ampules at Three Different Temperatures.*—In order to prevent sera from absorbing carbon dioxide during storage in the freezer, aliquots of serum were dispensed into chemically clean glass ampules and these were flame-sealed. Comparisons were made of the antibody titers of portions of sera stored in the ampules at room temperature, icebox temperature, and freezer temperature over a six-week period. Tables VI, VII, and VIII illustrate the results of this study.

In none of the instances, the results for which are presented in the earlier tables, did a significant number of sera fail to maintain their original titers over the six-week period. If larger numbers of sera had been available for these studies, perhaps significant numbers of sera would be found to show decreases in titer while stored in flame-sealed glass ampules. On the basis of these results, however, it would appear that storage in flame-sealed glass ampules is much more effective than storage in screw-capped tubes. The fact that fewer sera showed decrease in titer when stored in the ampules than when stored in screw-capped tubes lends support to the theory that the absorption of carbon dioxide and resulting drop in pH might be responsible for the decrease in antibody titers of sera stored in screw-capped tubes in the freezer. It should be mentioned, how-

ever, that prozone reactions were observed with three of the four antilymphogranuloma venereum sera stored in flame-sealed ampules in the carbon dioxide freezer.

TABLE VI. THE LENGTH OF TIME SERA STORED IN FLAME-SEALED GLASS AMPULES RETAINED THEIR ORIGINAL COMPLEMENT-FIXING ANTIBODY TITERS WHEN STORED AT ROOM TEMPERATURE

TYPE OF ANTIBODIES CONTAINED IN THE SERA	NUMBER OF SERA	NUMBER OF SERA WHICH RETAINED ORIGINAL ANTIBODY TITERS AFTER TIME INTERVALS OF: ONE WEEK	TWO WEEKS	THREE WEEKS	FOUR WEEKS	FIVE WEEKS	SIX WEEKS
Lymphogranuloma venereum	4	4	4	3	3	3	3
Influenza A	2	2	2	2	2	2	2
Influenza B	4	4	4	4	4	4	4
Total	10	10	10	9	9	9	9

TABLE VII. THE LENGTH OF TIME SERA STORED IN FLAME-SEALED GLASS AMPULES RETAINED THEIR ORIGINAL COMPLEMENT-FIXING TITERS WHEN STORED AT ICEBOX TEMPERATURE

TYPE OF ANTIBODIES CONTAINED IN THE SERA	NUMBER OF SERA	NUMBER OF SERA WHICH RETAINED ORIGINAL ANTIBODY TITERS AFTER TIME INTERVALS OF: ONE WEEK	TWO WEEKS	THREE WEEKS	FOUR WEEKS	FIVE WEEKS	SIX WEEKS
Lymphogranuloma venereum	4	4	4	4	4	4	4
Influenza A	5	5	5	5	5	5	5
Influenza B	4	4	4	4	4	4	4
Total	13	13	13	13	13	13	13

TABLE VIII. THE LENGTH OF TIME SERA STORED IN FLAME-SEALED GLASS AMPULES RETAINED THEIR ORIGINAL COMPLEMENT-FIXING ANTIBODY TITERS WHEN IN THE CARBON DIOXIDE FREEZER

TYPE OF ANTIBODIES CONTAINED IN THE SERA	NUMBER OF SERA	NUMBER OF SERA WHICH RETAINED ORIGINAL ANTIBODY TITERS AFTER TIME INTERVALS OF: ONE WEEK	TWO WEEKS	THREE WEEKS	FOUR WEEKS	FIVE WEEKS	SIX WEEKS
Lymphogranuloma venereum	4	4	4	3	2	2	2
Influenza A	5	5	5	5	5	5	5
Influenza B	4	4	4	4	4	4	4
Total	13	13	13	12	11	11	11

DISCUSSION

A knowledge of the optimal temperatures of storage for sera containing complement-fixing antibodies against viral and rickettsial diseases has several practical applications. First, a diagnosis of such diseases on the basis of complement fixation tests may be made only when a rise in antibody titer is demonstrated in the convalescent phase serum. Because discrepancies in antigens make it necessary to test the acute phase and convalescent phase serum in parallel against the same antigen, it is necessary that the acute phase serum be stored and tested again with the convalescent phase serum. If the conditions of storage permit a drop in complement-fixing titer of the acute phase serum, it might show a lower titer than the convalescent serum when the two are run

in parallel, and thereby lead to the erroneous conclusion that a rise in antibody titer had occurred during the course of the disease. Second, as few laboratories in the country now run complement fixation tests for viral and rickettsial diseases, it is usually necessary to ship specimens to distant laboratories. In these instances it is essential to know if the samples should be shipped under refrigeration or if they may be shipped by air mail without refrigeration. Another application of the knowledge of optimal temperatures for the storage of these sera is in the matter of maintaining stable antisera for standardizing complement-fixing antigens and for use as positive control sera in the complement fixation tests.

The results of these studies would indicate that in relation to the antigens tested, acute phase sera may be stored in screw-capped tubes in the icebox until they are run in parallel with convalescent phase sera if the length of time they are stored is no longer than three weeks. If they are to be kept for a longer period of time, up to and including six weeks, they should be stored in flame-sealed glass ampules in the icebox or in the carbon dioxide freezer. Our investigations also show that sera which need to be stored for only a short period of time (up to three weeks) before being run in diagnostic tests may be placed in screw-capped tubes in the icebox.

In these studies all of the sera stored at room temperature (regardless of the type of container) maintained their original complement-fixing antibody titers for at least two weeks. This period of time would allow shipment of sera by air mail to distant laboratories, at least in temperate climates, without any refrigeration. In these cases, however, utmost caution should be taken to keep the specimens sterile, as bacterial growth would undoubtedly cause the sera to become anticomplementary.

The results obtained in the investigation over a six-week period would indicate that positive control sera used to standardize complement-fixing antigens should be stored in flame-sealed glass ampules either in the icebox or in the carbon dioxide freezer. Further studies are being conducted to determine the effects of longer periods of storage under various conditions upon the complement-fixing antibody titers of human sera.

Any sera stored in the freezer should be dispensed in flame-sealed glass ampules rather than screw-capped tubes, as our investigations have shown that sera stored in the latter facility show a rapid decrease in titer. Why portions of serum stored in the carbon dioxide freezer show a decrease in complement-fixing antibody titers is not known. It was shown that the absorption of carbon dioxide resulted in a drop in pH of the serum, which is probably responsible rather than the low temperatures. Perhaps the low pH brings about denaturation of some of the antibody molecules, and these molecules may then be unable to combine with the homologous antigen or to form complement-fixing complexes with the antigen.

On the basis of the results obtained in this study it would appear that it is not the absorption of carbon dioxide which is responsible for the elicitation of prozones of sera stored in the carbon dioxide freezer. Perhaps the explanation is similar to the one proposed by Bawden and Kleczkowski[7] to account for

the appearance of prozones in complement-fixing sera against certain plant viruses when these sera were heated at temperatures of 75° C., 80° C., and 90° C. These workers postulated that complexes were formed between the antibodies and other serum proteins during heating which caused such interference with complement fixation.

SUMMARY

Studies were conducted to determine the optimal conditions for storage of human sera containing complement-fixing antibodies against the antigens of lymphogranuloma venereum, psittacosis, influenza A, and influenza B.

It was found that all human sera stored in screw-capped tubes maintained their original antibody titers when stored at room temperature (21° to 30° C.) for as long as two weeks, and at icebox temperature (4° to 10° C.) for at least three weeks. Sera stored in screw-capped tubes in the carbon dioxide freezer showed a rapid decrease in complement-fixing antibody titer. Fifty per cent of the sera tested lost their original titers after just one week's storage. Storage in the carbon dioxide freezer also caused some sera to show a prozone reaction.

Sera stored in screw-capped tubes in the carbon dioxide freezer were found to absorb sufficient carbon dioxide to lower their pH to 6.

Of the human sera stored in flame-sealed glass ampules, 90 per cent stored at room temperature retained their original titers for six weeks, 100 per cent stored at icebox temperature retained their original titers for six weeks, and 85 per cent stored in the carbon dioxide freezer retained their original complement-fixing antibody titers for six weeks.

Prozones developed in sera stored in the carbon dioxide freeezer regardless of the type of container in which they were stored.

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LABORATORY METHODS

PROPHYLAXIS OF EPIDEMIC PAROTITIS THE ANTIBODY RESPONSE FOLLOWING INJECTION OF MUMPS ANTIGEN IN VARYING DOSAGE

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INTRODUCTION

MUMPS continues to be a disease of importance among children and in military populations. Although its incidence in military practice is usually only 1.5 to 7 per 1,000,[1-2] it nevertheless tends to cause sporadic, prolonged epidemics.[3-7] Because of its complications and average duration of approximately two weeks in uncomplicated, and three to four weeks in complicated cases,[5] it remains a disease for which a suitable prophylactic agent is desirable.

A number of investigators[8-16] have developed and tested mumps vaccines by determining their effectiveness in stimulating the production of complement-fixation (CF) and hemagglutination-inhibition (HI) antibodies. In these reports, antibody titers increased following vaccination. Comparison of the results reported in various investigations indicates that there were wide variations in the test populations, methods used in preparation of vaccines, vaccination procedures, and serologic tests employed in measuring antibody responses.

The demonstrated effectiveness of the various vaccines in stimulating the production of antibodies led to clinical trials to determine their prophylactic value. Habel,[17] Henle and associates,[16, 19] and Stokes and colleagues[18] have reported that the incidence of the disease and the frequency of complications[20] were diminished in vaccinated populations.

Ultraviolet light has proved its effectiveness as an agent for inactivating viruses for use in vaccines.[21] It was our purpose to determine whether ultraviolet inactivated mumps virus retained its antigenicity. To test this hypothesis single injections of varied amounts of the vaccine were given to naval

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The opinions and assertions herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or the Naval Service at large.

The usage of commercially available products in connection with the project cannot be construed as indicating endorsement or approval of such products by the authors or by the Naval Service, nor as preference for one preparation of mumps vaccine to the exclusion of other similar products which are or may become commercially available.

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recruits. Subsequently, determinations were made of the CF antibody levels, their rate of emergence, and the proportion of serologically responsive subjects. The effects of prevaccination titers and past history were considered in the interpretation of results. The study was limited to 70 days since it was conducted in a transient military population. The data indicated that 48 to 240 CF units of vaccine stimulated the production of CF antibodies in significant amounts in 14 to 28 days with significant titers remaining 70 days after vaccination. In those with prevaccination titers of less than 2.0 and with negative histories of mumps, the greatest response followed the injection of 240 units.

PROCEDURES AND MATERIALS

*Preparation of the Vaccine.**—This was described in a previous publication.[22] Normal allantoic fluid control material was prepared by alcohol precipitation, but was not irradiated. Nitrogen concentration of the mumps vaccine (determined by the Kjeldahl method) was 0.19 mg./ml., and of the control material 0.038 mg./ml.

Selection and Grouping of the Subjects.—Twelve companies of naval recruits, of approximately 60 men each, were selected during the period in which they were receiving routine immunization against smallpox, typhoid fever, and tetanus.

On the first day of observation each recruit was questioned as to egg sensitivity and history of mumps. After bleeding for prevaccination CF titers, eight groups were formed from the alphabetically aligned men by strict rotation. A few of the individuals in Group 1 (Table I) were sensitive to eggs; these received 1.0 ml. of physiologic saline solution containing a 1:10,000 concentration of Merthiolate (Thimerosal, Lilly). These subjects served as controls in the event of an outbreak of mumps. The number of men in this group was increased to 140 in comparison with the usual 69 to 85 in the remaining groups. This was done by giving saline solution instead of allantoic fluid to all three control groups in four companies. In the other eight companies, Groups 2 and 3 received 0.6 and 1.2 ml., respectively, of alcohol-precipitated normal allantoic fluid. These controls were designed primarily to determine whether allantoic fluid stimulated the production of mumps antibodies. Groups 4 through 8 received 0.3, 0.6, 0.9, 1.2, and 1.5 ml. of the mumps vaccine (i.e., 48, 96, 144, 192, and 240 CF units). Skin tests were omitted, since they have been shown to stimulate the formation of antibodies.[23, 24] As an additional control, vaccine left at room temperature for about two hours during the inoculation period was titrated for concentration of CF antigen; no decrease occurred. Studies in our laboratories indicated that the antigen was stable at 4° C. for at least one year.[22]

Blood specimens were obtained again from all subjects at 28 and 42 days after vaccination, and from the men in four companies also at 7 and 14 days.

Since a considerable amount of antibody persisted at 42 days, a second experiment was conducted four months after the first in which a different group of recruits was bled before, and 35 and 70 days after the injection of 144 units of vaccine.

Collection and Shipment of the Sera.—Blood was drawn from the antecubital vein approximately four hours after the noon meal. The serum was removed and transferred to corked, sterile serologic tubes which were placed in an insulated chest containing dry ice, the temperature of which ranged between -10° and -30° C. The sera were shipped by air to the laboratory. On arrival the frozen sera were stored at approximately 4° C. and were titrated within two weeks. Laboratory tests indicated that these procedures did not alter the CF titer.[22]

The Complement-Fixation Reaction.—The procedure used was essentially that of Habel.[17] The viral or "V" antigen only was used, since the evidence of Henle and associates[24] indicated that use of the V antigen affords a more satisfactory index of response to mumps vaccines than does the "S" (soluble) antigen. Sera were inactivated at 60° C. for 30 minutes. One and one-third units of complement were used; 4 minimal units of anti-

*This vaccine was kindly supplied by Eli Lilly & Company, Indianapolis, Ind.

gen were employed in the antibody titrations; and 2 to 4 units of antibody were employed in the antigen titrations. Although the feasibility of using 2 plus (2+) CF titer end points was considered, the 4 plus (4+) end point was selected because of its clearer definition. Titers were expressed as the reciprocal of serum dilutions before the addition of the remainder of the system.

In order to determine the reliability of the CF test, 76 samples of sera from different individuals were selected at random, divided into two parts and sent to the laboratory as unknowns. The results indicated that 49 per cent of the replicate samples were of identical titer, 30 per cent one tube higher, and 7 per cent one tube lower. Of the remaining 14 per cent, 5 per cent were two tubes higher, and 7 per cent two, and 2 per cent three, tubes lower respectively.

In no tests were cross reactions observed between serum specimens and the normal allantoic fluid controls.

The inactivation of the sera at 60° C. for 30 minutes appeared to introduce no experimental error, since a test group of 60 specimens had comparable titers regardless of whether they had been heated at 56° C. or 60° C.

RESULTS

The Relationship Between History of Mumps and Prevaccination Complement-Fixation Titer.—Table I reveals that 466 of 697 (68 per cent) had histories of one attack of mumps. No histories of second attacks were reported. Of the 466 with positive histories, 141 (30 per cent) had CF reactions below 2.0 CF units; 127 of 231 (55 per cent) with negative histories had titers less than 2.0. Stated differently, a positive history of mumps was confirmed serologically in 70 per cent, whereas a negative history was rendered equivocal in 45 per cent by means of the same test. Despite this, the antibody responses following vaccination indicated that the groups with positive and negative histories were basically different.

Antibody Response Following Mumps Vaccination in Subjects Considered Without Regard to Prevaccination Titers.—The entire population was first considered without regard to initial CF levels, but with attention to history of mumps. This was done in order to obtain information on the response one might expect in a group of naval recruits, all of whom would probably be vaccinated in the event of an epidemic. Inspection of the data in Table II, in which the subjects had initial geometric mean CF titers of 1.3 to 2.8, shows that the maximal mean titers for the "total" group considered without regard to history was 61.2, and was approximately the same (60.9 and 62.0) in those with positive and negative histories, respectively. The maximal antibody response followed 240 CF units of vaccine regardless of a history of mumps. In subjects with positive histories of mumps, 144 to 192 units resulted in titers essentially equal to those which followed 240 units. In contrast, in those who had no prior history of mumps the lower doses of vaccine were followed by lower mean titers.

Time of Detection of Maximal Antibody Levels Following Vaccination.—The levels of antibody were observed in small groups of 4 to 28 men at 7 and 14 days and in larger units of 19 to 60 men at 28 and 42 days. These subjects were considered independently of prevaccination titers. Levels of 4.9 to 12.2 CF units of antibody were observed at 7 days in those with positive histories and 3.2 to 6.9 in those with negative histories. Men who had previous histories of mumps had a tendency to reach their maximum titers in 14 days regardless

TABLE I. COMPLEMENT FIXATION ANTIBODY TITERS FOR MUMPS VIRUS IN BLOOD SERA OF NAVY RECRUITS PRIOR TO INOCULATION WITH CONTROL MATERIAL OR MUMPS VACCINE ACCORDING TO PRIOR HISTORY OF MUMPS

INOCULUM		SALINE		ALLANTOIC FLUID				ULTRAVIOLET IRRADIATED, ALCOHOL PRECIPITATED MUMPS VACCINE										ALL GROUPS		TOTAL OF ALL GROUPS
GROUP NO.		1		2		3		4		5		6		7		8				
DOSE (ML.)		1.0		0.6		1.2		0.3		0.6		0.9		1.2		1.5				
DOSE (CF U.)		0		0		0		48		96		144		192		240				
HISTORY OF MUMPS		YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	
NO. OF MEN WITH	32			1	0							1	0					2	0	2
PREVACCINATION	16	2	0			3	0	2	0	2	0	1	0	2	0	2	0	14	0	14
CF ANTIBODY	8	14	3	2	0	3	0	3	1	9	0	8	0	10	2	9	1	58	7	65
TITER* OF	4	15	5	10	6	6	3	16	2	10	5	19	6	13	3	13	2	102	32	134
	2	25	21	21	9	19	6	17	6	17	7	13	2	20	7	17	7	149	65	214
	<2	25	30	11	11	16	13	23	15	17	17	19	14	11	15	19	12	141	127	268
TOTAL		81	59	45	26	47	22	61	24	55	29	61	22	56	27	60	22	466	231	697

*Expressed as reciprocal of dilution.

of the dosage of vaccine. Men with negative histories tended to reach maximum titers in 28 days following 192 to 240 CF units of vaccine. With smaller doses the antibody response was less and the maximal level was achieved in 14 days.

TABLE II. GEOMETRIC MEANS OF COMPLEMENT FIXATION ANTIBODY TITERS FOR MUMPS VIRUS IN SERA OF NAVY RECRUITS BEFORE AND AFTER VACCINATION WITH CONTROL MATERIALS AND MUMPS VACCINE ACCORDING TO A PRIOR HISTORY OF MUMPS

HISTORY OF MUMPS	DAYS AFTER INOCULATION	MEAN ANTIBODY TITER OF GROUPS* BY DOSAGE AND MUMPS ANTIGEN CONTENT OF INOCULUM							
		SALINE	ALLANTOIC FLUID		MUMPS VACCINE				
		1.0 ML.	0.6 ML.	1.2 ML.	0.3 ML.	0.6 ML.	0.9 ML.	1.2 ML.	1.5 ML.
		0 CF U.	0 CF U.	0 CF U.	48 CF U.	96 CF U.	144 CF U.	192 CF U.	240 CF U.
YES	0	2.5	2.2	2.2	2.1	2.5	2.6	2.8	2.5
	28	4.1	4.8	4.8	20.9	44.1	58.5	55.4	60.9†
	42	4.4	4.7	5.5	22.7	35.7	51.5	42.9	50.8
NO	0	1.6	1.7	1.3	1.5	1.5	1.6	1.6	1.6
	28	2.4	3.6	3.5	13.8	20.8	27.3	32.9	62.0
	42	2.5	2.8	3.9	15.1	17.8	25.7	24.3	51.4
TOTAL	0	2.1	2.0	1.9	1.9	2.1	2.3	2.4	2.3
	28	3.3	4.3	4.3	18.5	34.6	47.6	46.9	61.2
	42	3.5	3.9	4.9	19.5	28.4	42.1	35.7	51.0

*Number of men in group given in Table I.
†Highest titers obtained are underlined.

Antibody Response in Subjects with Prevaccination Titers of Less Than 2.0 CF Units and Negative Histories for Mumps.—Attention was turned to this group because these subjects were considered as most representative of the *de novo* immune status. Table III shows that the maximal mean CF antibody titer of those with negative histories having prevaccination titers less than 2.0 was 30.0 at 28 days and 32.0 at 42 days following the injection of 240 CF units of vaccine. Paradoxically, in those with positive histories, 144 and 192 CF units elicited higher CF antibody levels than 240; this was most noticeable in the 28-day titers.

TABLE III. GEOMETRIC MEAN COMPLEMENT FIXING ANTIBODY TITER FOLLOWING VACCINATION WITH MUMPS VACCINE ACCORDING TO THE PAST HISTORY OF MUMPS IN NAVY RECRUITS WHO HAD A PREVACCINATION TITER OF LESS THAN 2.0 CF UNITS

HISTORY OF MUMPS	DAYS POST-VACC.	DOSAGE OF MUMPS VACCINE							
		0.3 ml.		0.6 ml.		0.9 ml.		1.2 ml.		1.5 ml.	
		48	CF U.	96	CF U.	144	CF U.	192	CF U.	240	CF U.
POSITIVE	28	13.1	(21)*	30.7	(17)	61.7†	(19)	45.3	(10)	30.7	(17)
	42	20.8	(21)	26.0	(15)	39.8	(19)	32.0	(10)	30.8	(18)
NEGATIVE	28	8.0	(15)	13.5	(16)	20.5	(14)	23.2	(13)	30.0	(11)
	42	11.3	(14)	12.1	(15)	17.7	(14)	15.2	(13)	32.0	(10)
TOTAL	28	10.8	(36)	20.6	(33)	38.7	(33)	31.1	(23)	30.5	(28)
	42	16.3	(35)	18.0	(30)	28.2	(33)	21.0	(23)	31.2	(28)

*Number of subjects in group in parentheses.
†Underlined figures are highest titers in group.

Relation Between Pre- and Postvaccination Titers.—Data pertaining to the relation between pre- and postvaccination titers, in terms of the response to varying doses of vaccine, are presented in Table IV. Although information adequate

for comparative study of both 28 and 42 days was available, only the former was chosen. This course was adopted because the maximal titers were detectable at 28 days, with somewhat lower levels at 42 days. The data indicated that prevaccination titers greater than 4.9 were associated with consistently higher 28-day mean titers than were initial titers below 2.0. With initial titers of between 2.0 and 4.9, results with the several doses of vaccine were intermediate. It is noteworthy that the 28-day mean titers following injection of all the control materials increased slightly as the initial titers rose, and that the highest mean titer (12.7) was observed in the group with a prevaccination titer of >4.9 that received the greater amount (1.2 ml.) of allantoic fluid.

When the relationship of history to postvaccination titers was considered, it was found that those with positive histories attained higher titers than those who had no history of mumps.

TABLE IV. RELATIONSHIP OF COMPLEMENT FIXATION ANTIBODY TITERS FOR MUMPS ANTIGEN IN SERUM TWENTY-EIGHT DAYS AFTER RECEIPT OF CONTROL MATERIALS AND VARYING DOSES OF MUMPS VACCINE TO THE TITERS IN THE PREVACCINATION SERUM

TWENTY-EIGHT DAY POSTVACCINATION TITERS OF SUBJECTS RECEIVING	INOCULUM		INITIAL TITER* < 2.0	2.0-4.9	> 4.9
	MUMPS VACCINE	48 CF U.	10.8 (36)†	29.1 (22)	28.4 (23)
		96 CF U.	20.6 (33)	44.6 (23)	55.4 (24)
		144 CF U.	38.7 (33)	36.8 (15)	65.3 (34)
		192 CF U.	31.0 (23)	49.0 (26)	62.5 (29)
		240 CF U.	30.5 (28)	81.5 (23)	99.0 (27)
	SALINE SOLUTION	1.0 ML.	1.5 (50)	3.5 (44)	8.6 (38)
	ALLANTOIC FLUID	0.6 ML.	1.7 (19)	4.9 (28)	9.2 (18)
		1.2 ML.	2.5 (28)	4.2 (25)	12.7 (15)

*History ignored.
†Numbers of subjects in parentheses.

Incidence of Failures Among Those With Negative Histories and CF Titers Less Than 2.0—It seemed advisable to determine the incidence of failure of the several doses of vaccine to elicit antibodies in this group because a vaccine to be effective should stimulate significant levels of antibodies in a high percentage of the population. The CF levels at 28 days were used because the number of individuals available was adequate for study, and because the titers at this time were either at or near maximum. In order to avoid commitment to any particular titer as indicative of immunity, the data are presented in terms of failures to attain various antibody levels (Table V).

In the control subjects, the incidence of failures following injection of saline solution increased from 70 per cent at the 2.0 level of CF antibody response to almost 100 per cent at 4.0 and above. With both amounts of allantoic fluid used a pronounced difference from the saline group was seen in the incidence of failures at antibody levels of 2.0 and 4.0. Only at a titer of 8.0 did a failure rate of 90 to 100 per cent occur.

Special attention was given to the group which received 240 units of vaccine and which showed the maximal antibody response. No failures occurred in the group when tested at a titer level of 2.0 with a progressive increase of failures from 9 per cent at 4.0 units to 100 per cent at 2560. Results were

TABLE V. FREQUENCY OF FAILURE OF CONTROL MATERIALS AND SEVERAL DOSES OF MUMPS VACCINE TO PROVOKE VARIOUS LEVELS OF COMPLEMENT FIXATION ANTIBODY TITERS FOR MUMPS IN NAVY RECRUITS WHO HAD A NEGATIVE HISTORY OF MUMPS AND ANTIBODY TITER OF LESS THAN 2.0 IN THE PREVACCINATION SERUM

INOCULUM AND DOSE		NO. OF MEN	PER CENT WITH TWENTY-EIGHT-DAY POSTVACCINATION TITERS BELOW								
			2.0	4.0	8.0	16.0	32.0	64.0	128.0	256.0	512.0
SALINE SOLUTION	1.0 ml.	27	70	96	100	100	100	100	100	100	100
ALLANTOIC FLUID	0.6 ml.	11	54	82	91	100	100	100	100	100	100
	1.2 ml.	13	23	69	92	92	100	100	100	100	100
MUMPS VACCINE	48 CF U.	15	13	27	47	53	67	93	100	100	100
	96 CF U.	16	6	25	25	44	69	69	100	100	100
	144 CF U.	14	7	7	14	21	57	71	93	93	100
	192 CF U.	13	8	8	15	23	38	69	85	100	100
	240 CF U.	11	0	9	18	27	36	45	64	100	100

similar with 192 units of vaccine. The difference in the incidence of failure in terms of the dosage factor became more apparent with the employment of the higher titers as measures of antibody response. Thus, when a level of 64.0 was used, the response following 48 units of vaccine was 93 per cent failures, with a decrease to 45 per cent failures after 240 units.

Duration of the Antibody Response.—Since the mean titers had not decreased markedly in 42 days in the first recruits studied (Table II), a second group (Table VI) was injected with 144 units of vaccine, and CF antibody levels were determined at 35 and 70 days. This group likewise received concurrent typhoid, tetanus, and smallpox immunizations. With only one exception, pronounced decreases in antibody titers were not observed between the thirty-fifth and seventieth days. The exception was in the group with both positive histories and initial mean titers 6.2 to 6.7, wherein a change of about 23 CF units from the highest titer, 59.8, to 37.0 occurred between 35 and 70 days. If both history and initial titer are ignored, the entire population of 105 men had a mean titer of 26.9 at 35 days and one of 21.0 at 70 days. In reference to history and prevaccination titers, the lowest results were observed in those with negative histories and prevaccination titers below 2.0, with 35 and 70 day mean titers of 9.9 and 6.6, respectively. The group with prevaccination titers of 2.0 showed an intermediate response, with little change between 35 and 70 days. The uninjected controls showed relatively little change.

DISCUSSION

In the early stages of this research the complement-fixation test was selected as a test of serologic response because others[8, 13, 14, 15, 25] considered it satisfactory. Subsequently, Habel[20] criticized its use and preferred the neutralization test. Bashe and associates[26] also preferred the neutralization test but indicated that the complement-fixation test remained a useful procedure because of its speed and simplicity, and provided that titers of 16.0 or greater were attained. It should be pointed out, however, that Henle and associate[16] reported that a high percentage of his subjects were immune at the 1:8 level. Apparently prevalent opinion concurs that the expensive and time-consuming neutralization test is a better indication of immunity than the CF.

TABLE VI. THE COMPLEMENT FIXATION ANTIBODY TITER FOR MUMPS ANTIGEN 35 AND 70 DAYS AFTER THE INJECTION OF 144 CF UNITS OF VACCINE IN NAVY RECRUITS ACCORDING TO THE PAST HISTORY OF MUMPS AND THE ANTIBODY TITER PRIOR TO VACCINATION

HISTORY	DAYS POSTVACC.	ANTIBODY TITER FOR MUMPS ANTIGEN						TOTAL ALL SUBJECTS	
		INITIAL TITER < 2.0		INITIAL TITER 2.0		INITIAL TITER 6.2–6.7			
		CONTROL*	VACCINE	CONTROL	VACCINE	CONTROL	VACCINE	CONTROL	VACCINE
POSITIVE	0	1.0 (15)†	1.0 (11)	2.0 (17)	2.0 (28)	6.2 (30)	6.4 (21)	2.9 (62)	2.6 (60)
	35	1.2 (17)†	12.9 (13)	1.6 (20)	36.3 (28)	3.2 (32)	59.8 (24)	2.1 (69)	35.7 (65)
	70	1.2 (15)	7.1 (11)	2.2 (17)	36.9 (29)	4.3 (30)	37.0 (24)	2.6 (62)	27.8 (64)
NEGATIVE	0	1.0 (24)	1.0 (21)	2.0 (7)	2.0 (10)	6.7 (9)	5.9 (9)	1.7 (40)	1.8 (40)
	35	1.0 (25)	9.9 (23)	2.0 (8)	24.9 (11)	2.1 (10)	41.8 (13)	1.4 (43)	18.3 (47)
	70	1.0 (25)	6.6 (21)	1.8 (8)	20.6 (11)	3.4 (9)	43.5 (9)	1.5 (42)	13.5 (41)
TOTAL	0	1.0 (39)	1.0 (32)	2.0 (24)	2.0 (38)	6.4 (39)	6.2 (30)	2.4 (102)	2.3 (100)
	35	1.1 (42)	10.9 (36)	1.7 (28)	33.0 (39)	2.9 (42)	52.7 (37)	1.8 (112)	26.9 (112)
	70	1.1 (40)	6.7 (32)	2.1 (25)	31.5 (40)	4.1 (39)	38.7 (33)	2.1 (104)	21.0 (105)

*Unvaccinated subjects.

†Apparent discrepancies due to loss of sera in handling.

Our data on random samples sent as unknowns to the laboratory indicated that 86 per cent of replicate samples agreed within a one tube range. It is relevant that Henle and colleagues,[16] without tabulating their data, reported close agreement of the results of repeated tests.

There was a fair degree of relationship between history of mumps and CF titers. Seventy-five per cent of those with positive and 41 per cent of those with negative histories, or 62 per cent of the total group, had CF antibody titers of 2.0 or above. Henle and associates[25] reported similar results. As to the relationship between CF titers of 2.0 or more and a history of mumps, the incidence in our study (75 per cent) is in substantial agreement with that of Henle, i.e., 71 per cent. However, of those with CF titers less than 2.0, Henle's Philadelphia subjects had 35 per cent with positive histories, whereas our group has 52 per cent. The reasons for this difference are not clear.

Henle and colleagues[25] have stated that neither noninfected embryonated egg materials nor antigens of unrelated infectious agents are satisfactory as control substances. The controls employed in our investigation indicated that postvaccination titers were within the same range as the prevaccination levels. The single exception was the group with prevaccination titers of 4.9 or higher given the large amounts of allantoic fluid control material, wherein the 28-day mean postvaccination titer was 12.7 (Table IV), possibly indicative of a nonspecific stimulus. The nitrogen content of our control material was about one-fifth that of the vaccine, although both materials had been prepared by the same method. It might be argued that quantitatively the nitrogen content of the control material and vaccine should have been comparable; however, a qualitative difference would have remained.

No clinical cases of mumps appeared in the trainees studies, although 6 appeared in other personnel during the period of the investigation at the Training Center. Therefore, the controls served a second purpose, i.e., detection of the possible subclinical antigenic activity of the mumps virus. Inasmuch as only small antibody rises appeared in all the controls, except in those with initial titers of 4.9 or higher given 1.2 ml. of allantoic fluid, it can be assumed that the increases in titer following vaccination were the result of the antigen of the vaccine, and not of subclinical mumps.

An estimation of the possible protective value of the vaccine was based on the study of Henle and associates[16] in which the incidence of the clinical disease eight to eleven months after vaccination was related to CF titers observed four to five weeks following vaccination. It is quite possible that the CF titer is not as satisfactory an indication of immunity following vaccination as the neutralization titer[16, 20] and that comparisons between two laboratories are subject to error. In the absence of a test of the vaccine in the prevention of the disease, however, it seemed that a comparison was indicated. Since the most important use of the vaccine was considered to be the immunization of those in the *de novo* status, attention was directed to those with prevaccination titers less than 2.0.

In the study of Henle and associates,[16] it was observed that in those with initial titers below 2.0, independent of history, a CF titer of 8.0 was protective

in 84 per cent and a titer of 16.0 in 90 per cent of the population. Our subjects with prevaccination titers less than 2.0 developed mean titers of 30.0 following 240 units of vaccine in our first group of recruits (Table III), and titers of 9.9 in the second group following 144 units (Table VI). It would appear from these results that the CF antibody levels reached with the vaccine used in these studies were probably in the protective range.

It is noteworthy that in the study conducted four months later (Table VI) subjects with prevaccination titers of less than 2.0 given suboptimal doses of vaccine developed a mean titer of 9.9 in 35 days, and this was but slightly influenced by history. The latter result, definitely lower than that observed in the first experiment (Table III), but still in a protective range, is difficult to explain, but may simply reflect the possibility that groups as well as individuals may vary widely in antibody response. The possibility that the vaccine had lost potency seems remote since its CF titer and CF antibody stimulating ability in guinea pigs have been demonstrated to be stable.[22]

The influence of history on the postvaccination titers was reflected in two ways: (1) higher postvaccination titers appeared among those with positive histories, except when a dose of 240 units was used, wherein the results were about equal; (2) practically maximal mean titers (58.4) were attained with a lower dose (144 units) among those with positive histories whereas 240 units were required to reach this level in those with negative histories. This difference was manifest whether the group as a whole (Table II) or those in the so-called *de novo* immune status (Table III) were considered. It would appear that the anamnestic reaction was more pronounced in those with positive histories.

Since, as already indicated, a CF titer of 8.0 was considered protective, we designated a level of 4.0 or less a failure. In our subjects (Table V), among those with negative histories and CF titers of less than 2.0 given 144 to 240 units of vaccine, 18 per cent were failures when tested at 28 days.

Results pertaining to the persistence of antibody titers beyond a period of 70 days could not be obtained in this study. Our data (Table VI) indicate that at 35 days all groups, regardless of initial titer or history, had a mean titer in the protective range. At 70 days a tendency was noted for those with *de novo* immune status to approach antibody levels which may be considered nonprotective.

Although a number of studies on mumps vaccination have been reported,[8-20] a comparison of our results with those of Henle and colleagues,[16] was made because of similarities in methods of preparation and testing of the vaccines. In their investigation among a group of 149 adults, without reference to dose, type of vaccine, or initial titer, a mean CF titer of 45.0 appeared in 14 days. Our subjects had a mean titer of 65.7 in the same period and after 144 units. In those with titers less than 2.0 our highest mean titer at 14 days in a small group after 144 units was 57.0, whereas one of 33.0 was observed by the Henle group.

Henle and colleagues[16] investigated four different vaccines concentrated by alcohol precipitation or centrifugation and inactivated by formalin or

ultraviolet light in various combinations of methods. Their vaccines represented a 16- to 20-fold concentration of the original virus-containing allantoic fluid. The vaccine used in this study, prepared by alcohol precipitation and ultraviolet light inactivation, consisted of a fivefold concentration of infected allantoic fluid. Henle's vaccine was titrated by the hemagglutination reaction, whereas the potency of our antigen was measured by complement-fixation. Since the titrations of the sera were not strictly comparable, no precise comparisons can be made. It is noteworthy, however, that approximately 1.0 ml. of our vaccine elicited mean titers which were in the range of those stimulated by 4.0 ml. of the twentyfold concentrate, i.e., about 1/16 as much of our ultraviolet inactivated vaccine was required to produce about the same response as Henle's. It is possible that smaller amounts of Henle's vaccine would also have been as effective. Other explanations for this difference might be that the virus in our unconcentrated allantoic fluid was of higher titer than the Henles' or that the antigen was more stimulative.

It has been pointed out by Bashe and associates[26] that failures with present vaccines occur too frequently. They suggested higher dosages, the use of adjuvants, or 2 or 3 injections at suitable intervals. The fact that 18 per cent of our so-called susceptible subjects were considered vaccine failures (Table V) would support the view that improvement is necessary. Our data indicate that antigen prepared by ultraviolet light irradiation and alcohol precipitation might be effective in relatively low concentration and should be studied further.

As to untoward reactions to mumps vaccine, Habel[20] encountered one anaphylactic reaction among 1,344 subjects injected with chick embryo vaccine in beeswax peanut-oil adjuvant. In all his subjects there were deep subcutaneous nodules noticed for several weeks after vaccination, but no evidence of sensitization or of local reactions was noted in a group of 383 given a second dose of the vaccine. In a group of 65 subjects studied by Henle and colleagues,[16] only 4 had a fever of 100° F. or more, 3 malaise, 34 soreness, and 1 erythema. Following the injections of our vaccine there were no recorded constitutional or local reactions among the naval recruits. In vaccinations of medical students using the same vaccine, however, 3 of 20 reported generalized aching and febrile sensations within the first twenty-four hours after injection of a single dose of 144 units.

Hyperimmune mumps serum or mumps immune serum gamma globulin[27] have been recommended for the prevention of complications. Habel[28] has recommended that those showing early skin reactions to the mumps antigen be given passive prophylaxis since these individuals tend to develop complications. Because of these findings, our data (Table IV) were studied to determine the circumstances conducive to the emergence of maximal titers. It was observed that maximal titers were attained in those having initial antibody titers of >4.9 following 240 CF units of vaccine. Whether higher titers might have been attained with larger amounts of vaccine was not determined.

SUMMARY

Single subcutaneous injections of physiologic saline solution, varied amounts of alcohol-concentrated normal allantoic fluid and several amounts of alcohol-precipitated ultraviolet-inactivated mumps vaccine were given to groups of naval recruits.

Subjects with negative histories and prevaccination complement-fixation antibody titers of less than 2.0 responded with a maximal geometric mean titer of 32.0 28 days after the injection of the greatest amount of vaccine tested, i.e., 240 CF units. These individuals were considered as approximating the *de novo* immune status.

Those with prevaccination titers of 2.0 and higher, considered without reference to history, had maximal mean CF titers of 81.5 to 99.0 twenty-eight days after 240 units of vaccine. In general, those with positive histories responded with CF titers higher than those with negative histories.

Antibodies appeared in low concentration 7 days following vaccination, reached high levels at 14 and 28 days, and persisted at significant, though lower, levels for the duration of the study, i.e., 70 days.

The incidence of failure of the vaccine to stimulate CF antibody levels shown to be protective by others, was 14 to 18 per cent respectively when 144 to 240 CF units of vaccine were given.

It appears that 1.5 ml. of a fivefold concentrated, alcohol-precipitated, ultraviolet-inactivated mumps vaccine prepared from the allantoic fluid of embryonated eggs is a suitable antigen for the stimulation of CF antibodies in man. One-sixteenth the amount of this vaccine was required to elicit antibody responses comparable to those reported following use of vaccines prepared by other methods. Thus, it seems probable that the vaccine will prove as effective in the prevention of mumps, as have been other vaccines, offering the advantage of a smaller virus unitage and total volume of inoculum with attendant lower cost. However, further studies are indicated to reduce the incidence of failures.

Members of the staff of NAMRU-4 participating in this study were as follows: Lieutenant L. E. Anthony, Medical Corps, United States Navy, under whose direction the vaccinations and bleedings were carried out; Lieutenant C. E. Curtis, Medical Service Corps, United States Navy, Miss Darlene L. Phillips, and Mrs. Ruth A. Dmytro of the Biostatistics Department who tabulated the data; Robert L. Woolridge, York E. Crawford, Gilbert Rossner, and HM1 Joseph Cecil of the Department of Immunology who were responsible for the bleedings and preparatiin of the sera. Others who aided in the study were Paul Frank, HMC W. F. Haines, HMC Billy G. Cook, HM1 Walter R. Conley, HM1 I. A. Phillips, and HM2 Robert D. Parpart.

Particular appreciation is due Captain K. L. Nutting, Commanding Officer of the Recruit Training Command, and the members of his staff for the excellent cooperation afforded us in scheduling the recruit companies for vaccinations and bleedings.

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AN IMPROVED METHOD FOR DETERMINING PROPORTIONAL DISTANCES BETWEEN DILUTION TUBES IN THE HEMAGGLUTINATION-INHIBITION TEST

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IN 1942, Hirst and Pickels[1] described a photoelectric method for measuring optical density in titration tubes of the hemagglutination-inhibition test. Their method determines the point where 50 per cent of the cells have settled out and 50 per cent have remained in suspension after a seventy-five minute incubation period.

To determine the 50 per cent end point between two dilution tubes, it is necessary to calculate the proportional distance between them. This proportional distance is obtained by either an algebraic formula[1] or by means of a nomogram.[2]

To facilitate the calculation of titers in the performance of hemagglutination-inhibition tests for influenzal antibodies, a simple table (Table I) was prepared to be used in lieu of the nomogram. As the spectrophotometer is read by the serologist, the readings are recorded by an assistant, who can immediately obtain the proportional distance in the form of a decimal from the table. This method is more accurate because various recorders will read the same decimal instead of depending on their subjective observations of the straightedge on the nomogram.

DESCRIPTION OF THE TABLE

Numbers from 1 through 49 were placed across the upper and lower margins of the table. These represented the spectrophotometric readings (in per cent of light transmission through the titration tubes) which fall below the 50 per cent end point. Columns of numbers from 51 through 100 were then placed beneath figures 1, 10, 20, 30, 40, and 49 in the upper line. These columns are the tube readings above the 50 per cent end point. Decimal boundaries for each combination of lower and higher tube readings were determined by both the algebraic formula and the nomogram. The boundaries or limits of each decimal area were marked on the face of the table by irregular lines. The decimals for each area were then placed in the margin of the table.

USING THE TABLE

When the end point on the spectrophotometer is set at 50 (in per cent of light transmission through the control tube) paired readings will be obtained where the numbers are on either side of 50. For example, if these paired readings were 34 and 63, the proportional distance would be obtained from the table

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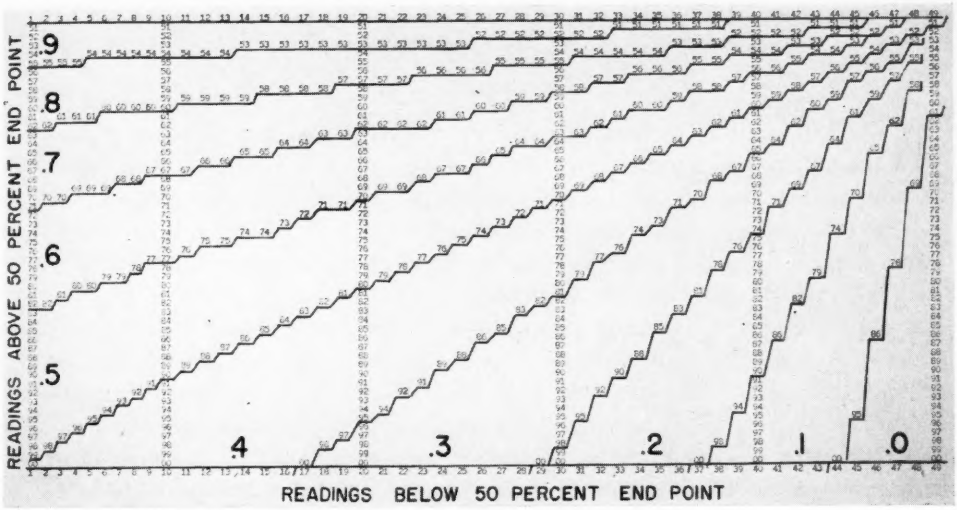
as follows: follow the top line to 34 and, using the column of figures under 30 as a guide, find 63 in the vertical column under 34. Reference to the margin shows that the combination 34-63 has a value of 0.5.

TABLE I. DETERMINING PROPORTIONAL DISTANCES BETWEEN DILUTION TUBES IN HIRST HEMAGGLUTINATION-INHIBITION TESTS



SUMMARY

A method is described which facilitates the calculation of titers in the performance of hemagglutination-inhibition tests for influenzal antibodies. It consists of a simple table which is used in lieu of the nomogram.[2] Decimals representing the proportional distance between dilution tubes are obtained from the table immediately after the recorder receives the paired titration tube readings. More consistent determinations are possible because of the elimination of subjective nomogram observations.

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INDIRECT ESTIMATION OF ALVEOLAR CARBON DIOXIDE TENSION

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MUCH attention has been focused in recent years on regulating ventilation in patients in respirators.[1] One helpful index is to maintain a particular tension of arterial carbon dioxide, which in turn is inversely proportional to the alveolar ventilation. Although man can adjust his acid base balance to various carbon dioxide tensions,[2] the sensitivity of his respiratory center varies inversely with this tension.[3] Thus, a patient who has been subjected to twice normal alveolar ventilation in a respirator for several days and who has a normal arterial pH will have to maintain this high ventilation for a considerable time when taken out of the respirator to prevent an otherwise severe respiratory acidosis. This problem becomes important when trying to wean patients from the respirator: the smaller the ventilatory demands made by the respirator, the easier the weaning. At one large respirator center many patients were encountered who had high ventilatory demands and very low alveolar P_{CO_2} values. In these subjects any attempt to reduce the ventilation by adjusting the respirator resulted in air hunger, headache, and many other symptoms, which delayed the restoration of normal P_{CO_2} in some cases for months.[4]

Frequent determinations of arterial blood gas tensions are uncomfortable for the patient, time-consuming for the hospital staff, and require trained laboratory personnel. An alternative for frequent, routine use is the measurement of alveolar or end-tidal carbon dioxide tension, which in the normal lung closely approximates the arterial carbon dioxide tension.[5] Unfortunately, there are no automatic carbon dioxide analyzers which are relatively cheap and stable, and which require no calibration or attention. This lack is a handicap for small hospitals which handle only occasional respirator patients. Any method would be helpful which would permit the approximate estimation of end-tidal carbon dioxide levels with simple and relatively stable instruments.

With this in mind, two relatively simple methods for the indirect estimation of alveolar CO_2 were investigated. One requires the use of the relatively stable Pauling oxygen tensimeter,* while the other requires an ordinary basal metabolism machine. Both methods are easily adaptable to routine ward use by a technician.

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MATERIALS AND METHODS

A. *The Oxygen Tensimeter Method.*—The alveolar O_2 tension is measured with the aid of a Rahn end-tidal sampling device* (Fig. 1, *A*).[6, 7] The CO_2 tension can be calculated if one is willing to assume that R, the respiratory quotient, will not vary far from a given average (in this study 0.81) and that if it is varied, for example, by sudden hyperventilation in a respirator, it may be expected to return to the previous level after thirty to forty-five minutes of steady ventilation at the new rate.[7]

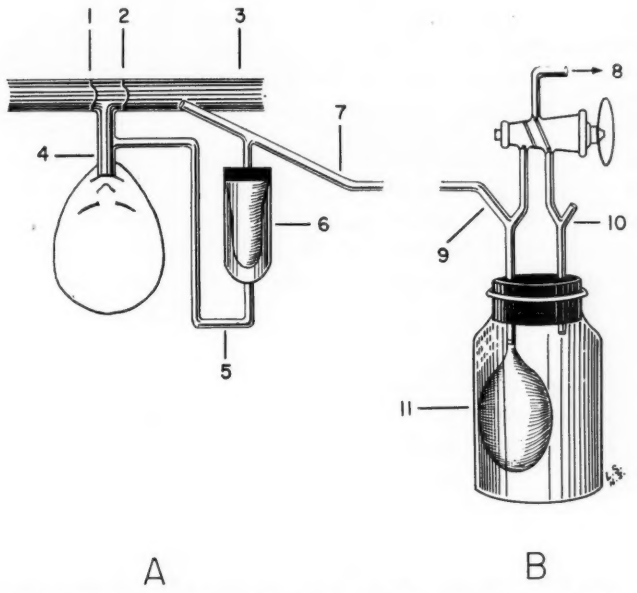

Fig. 1.—*A*, End-tidal air sampling device adapted for respirator patient: *1*, Intake valve; *2*, output valve; *3*, expired air hose; *4*, mouthpiece; *5*, balloon chamber hose; *6*, balloon chamber; *7*, end-tidal sampling hose.

B, Apparatus for collecting pooled sample of end-tidal air sufficiently large for the application of Behrmann's method: *8*, Hose leading to mercury-filled sampling tubes or gas analyzing apparatus and suction pump; *11*, anesthesia bladder. (A good rubber anesthesia bladder is adequate if the sample is to be analyzed immediately; otherwise, a bladder impervious to CO_2 must be substituted.) With stopcock in position shown and a clamp at *10*, end-tidal air is collected in the anesthesia bladder. By reversing stopcock and transferring clamp from *10* to *9*, bladder is emptied for washing or analysis of sample.

1. *Method for clinical use:* The subject breathes room air through an end-tidal air sampling device. End-tidal air is aspirated continuously without drying through a Pauling oxygen analyzer by means of a suction pump adjusted with the aid of a flowmeter to a rate of 100 ml. per minute or by a 50 ml. syringe and three-way stopcock. The reading of $P_{A_{O_2}}$, the end-tidal O_2 tension taken from the analyzer, is converted to terms of BTPS by the factor $\frac{P_B - 47}{P_B - P_{H_2O}}$ where P_B is barometric pressure in millimeters of mercury and P_{H_2O} is water vapor pressure at ambient temperature. $P_{I_{O_2}}$, the O_2 tension of inspired room air at BTPS, is 0.209 (P_B - 47) mm. Hg.

For a subject with an R of 0.81 breathing room air, the alveolar air equation[8] may be arranged as follows and solved for $P_{A_{CO_2}}$, the alveolar CO_2 tension in mm. Hg BTPS.

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$$P_{A_{CO_2}} = 0.844\ (P_{I_{O_2}} - P_{A_{O_2}}) \text{ ----------(1)}$$

(0.844 is the value of $\frac{R}{1 - 0.209\ (1 - R)}$ in this instance.)

The error in calculating $P_{A_{CO_2}}$ will be a function of the discrepancy between the actual and assumed values of R.

2. *Evaluation of reliability of the method:*

(a) *Experiment 1.* The apparatus described above was modified by interposing a mercury-filled sampling tube between the end-tidal sampling device and the pump, permitting the collection of a 50 ml. sample of end-tidal air over a period of about twenty seconds. In some cases a bladder apparatus (Fig. 1, *B*) was interposed instead, permitted the collection of a 750 ml. sample over a period of five minutes, an aliquot of which was promptly transferred to a sampling tube. Forty samples of end-tidal air were thus obtained: 29 from subjects and patients breathing quietly at rest and 11 from a poliomyelitis patient in a respirator. All were breathing room air. Each sample was analyzed in duplicate in the Scholander gas analyzer for oxygen and carbon dioxide concentrations, from which the tensions at BTPS were calculated. (The oxygen tensions read from the Beckman meter were ignored, in order to avoid the additional error introduced by breath-to-breath variations in tension and respiratory quotient which even the trained subject cannot overcome.) $P_{A_{CO_2}}$ was then calculated by equation (1). This will be referred to hereafter as the "calculated $P_{A_{CO_2}}$." The term "measured $P_{A_{CO_2}}$" will be used to refer to the values obtained by direct analysis on the Scholander.

(b) *Results:* The averages of the results of the 40 samples are summarized in Table I. On the average, the calculated $P_{A_{CO_2}}$ agrees very well with the measured $P_{A_{CO_2}}$. This merely reflects the fact that the mean measured value of R. Q. was taken as the assumed value. However, the discrepancy in individual cases between the measured and calculated $P_{A_{CO_2}}$ was moderate. (Mean ± 2 S.D. = 0 ± 6 mm. Hg.)

B. *Use of the Metabolism Apparatus.*—A form of the ventilation equation[8] may be stated thus, for subjects breathing any oxygen mixture free of CO_2.

$$P_{A_{CO_2}} = \frac{\dot{V}_{O_2} \times R \times (P_B - 47)}{\dot{V}_E - \dot{V}_D} \text{ ----------(2)}$$

where $P_{A_{CO_2}}$ is alveolar CO_2 tension in mm. Hg BTPS

$\dot{V}_{O_2}$ is oxygen consumption

where $\dot{V}_E$ is minute ventilation

$\dot{V}_D$ is dead space ventilation

provided $\dot{V}_{O_2}$, $\dot{V}_E$ and $\dot{V}_D$ are all expressed in similar units under similar conditions of temperature and pressure.

$\dot{V}_{O_2}$, respiratory rate, and a close approximation to $\dot{V}_E$ may be measured with a basal metabolism machine. If values for R and for the physiologic dead space are assumed, $P_{A_{CO_2}}$ may be calculated.

1. *Method for Clinical Use.*—A metabolism record is made, not necessarily under basal conditions. $\dot{V}_{O_2}$ is measured in the usual way, but not corrected from ATPS to STPD. $\dot{V}_E$ is computed in milliliters at ATPS by adding together the *inspiratory* tidal volumes (which gives

a closer approximation to the expired minute volume than the measurement of expiratory tidal volumes after CO_2 absorption). The respiratory rate is measured from the tracing.

To calculate $P_{A_{CO_2}}$ from the data thus obtained, one must make two assumptions: first, as in the previous method, that the R. Q. will not vary far from a given average (0.81); and second, that the physiologic dead space will approximate a given average. The values selected for this study were 150 ml. BTPS for men and 110 ml. BTPS for women. These are converted to ATPS by the factor $\frac{273 + T}{310} \times \frac{P_B - 47}{P_B - P_{H_2O}}$ where P_{H_2O} is water vapor pressure at ambient temperature T degrees centigrade. The volume of the mouthpiece employed is measured and added to the assumed physiologic dead space expressed at ATPS. The resulting volume is multiplied by the minute respiratory rate to give the dead space ventilation $\dot{V}_D$ in cubic centimeters per minute at ATPS Equation (2) may then be solved.

TABLE I. COMPARISON OF "MEASURED $P_{A_{CO_2}}$" WITH $P_{A_{CO_2}}$ CALCULATED BY THE ALVEOLAR AIR EQUATION AND BY THE VENTILATION EQUATION

			NUMBER OF MEASUREMENTS	$P_{A_{CO_2}}$ "MEASURED"	$P_{A_{CO_2}}$ "CALCULATED"	DIFFERENCE BETWEEN CALCULATED AND MEASURED VALUES IN INDIVIDUAL INSTANCES
Alveolar air equation. Experiment 1.			40	37 ± 7	37 ± 8	0 ± 6
Ventilation Equation	Experiment 2. Closed circuit method	Combined	28	38 ± 6	37 ± 11	0 ± 9
		Men	15	39 ± 6	38 ± 10	−1 ± 8
		Women	13	36 ± 4	36 ± 12	0 ± 12
	Experiment 3. Open circuit method		8	35 ± 5	36 ± 9	1 ± 12

$P_{A_{CO_2}}$ is expressed to the nearest millimeter Hg BTPS. The values are averages for each group ± 2 S. D. In the last column, the values are algebraic means ± 2 S. D.

2. *Evaluation of Reliability of the Method.*—

(a) *Experiment 2:* To test the range of error likely to be encountered in the use of this method, metabolism records were made on normal subjects alternating with collection of end-tidal air samples for direct analysis of alveolar carbon dioxide. The mouthpiece of a spirometer was replaced by an end-tidal sampling device. By clamping off the balloon chamber hose and the end-tidal sampling hose (Fig. 1), oxygen consumption and ventilation could be determined as described previously. By removing the clamps, end-tidal air could be collected over a period of five minutes in a bladder apparatus, and an aliquot transferred promptly to a sampling tube, for duplicate analysis in the Scholander gas analyzer.

Ten normal adults were employed: five men and five women. After a preliminary quiet rest on the table, the subject was connected to the mouthpiece of the sampling device and the spirometer filled with oxygen. End-tidal air was collected for five minutes and a sample transferred to a sampling tube. A metabolism record was made for six minutes immediately following the collection period. The "measured $P_{A_{CO_2}}$" was calculated from the CO_2 concentration of the sample as measured in duplicate in the Scholander gas analyzer. The "calculated $P_{A_{CO_2}}$" was calculated from the metabolism record as described previously, equation (2), and compared with the "measured $P_{A_{CO_2}}$." In each experiment, the procedure just described was repeated once or twice, yielding two or three pairs of values for $P_{A_{CO_2}}$.

(b) *Results:* The average results of 10 experiments, yielding 28 pairs of values for $P_{A_{CO_2}}$, are given in Table I: 15 pairs of values are from men, and 13 from women. The

average "calculated $P_{A_{CO_2}}$" agrees, within less than 0.5 mm. Hg, with the averaged "measured" value; but the discrepancy between the two in individual cases is considerable (mean ± 2 S. D. = 0 ± 9 mm. Hg).

(c) *Experiment 3:* The reliability of the method involving the ventilation equation was further analyzed as follows: A modification of the open circuit method for measuring ventilation and oxygen consumption was used. The Rahn end-tidal air sampling device was substituted for the conventional mouthpiece, so that end-tidal air could be collected in a bladder at the same time that mixed expired air was collected in the Douglas bag. While the subject was breathing room air, end-tidal air was collected at 150 ml. per minute and expired air collected in a Douglas bag for five minutes simultaneously. Samples of each were analyzed for CO_2 and O_2 in duplicate in the Scholander gas analyzer. The volume of expired air was measured in a Tissot spirometer and the volume and composition of expired air were corrected for the effect of the end-tidal sampling device.[9]

Minute ventilation, dead space, alveolar ventilation, oxygen consumption, and R. Q. were calculated from the data obtained. $P_{A_{CO_2}}$ was calculated directly from the end-tidal air analysis and indirectly by equation (2) employing both assumed and measured values of R. Q. and dead space.

The experiment was performed five times in three normal men and three times in a woman patient with poliomyelitis.

(d) *Results:* Results of the eight experiments are shown in Table I. When measured R. Q. and dead space (calculated by the Bohr equation) were employed, "measured" and "calculated" $P_{A_{CO_2}}$ agreed exactly to the nearest millimeter of mercury in six instances and within 1 mm. in two instances. When the usual assumed values of R. Q. and dead space were employed, the discrepancy between measured and calculated $P_{A_{CO_2}}$ was of the same order and distribution obtained by Experiment 2. The average calculated dead space of the men was 146 ml. BTPS, ranging from 113 to 171 ml., and the average value in the woman 110 ml. BTPS, ranging from 82 to 144 ml.

DISCUSSION

Neither of the two approaches here suggested offers exact estimation of the end-tidal carbon dioxide tension. The first method, based upon the alveolar air equation, yielded results in our subjects which were reliable within plus or minus 3 mm. Hg in two-thirds of the cases or within plus or minus 6 mm. Hg in 95 per cent of the cases. This degree of reliability is adequate for detecting gross instances of overventilation. It seems reasonable to offer the method for more extensive trial in poliomyelitis patients. If the range of error were no greater than the present studies indicate, it could be recommended as having distinct, but limited, value in institutions where more elaborate facilities were unavailable. It has two disadvantages: first, it requires special equipment; and second, there is at present no end-tidal sampling device designed for use in a patient with a tracheotomy.

The second method, applying the ventilation equation to data obtained with a Benedict-Roth spirometer, has the advantage that it requires no special equipment. However, in our experiments only two-thirds of the calculated values lay within 5 mm. Hg of the measured value, and 95 per cent lay within 9 mm. Hg. This is a more considerable error than the other method involves. The accuracy of the method depends upon the accuracy of prediction of the dead space and R. Q. This was demonstrated in Experiment 3 by making the indirect calcula-

tion employing the measured values for these variables; and indeed, if dead space is obtained by the Bohr equation, agreement between measured and calculated $P_{A_{CO_2}}$ is a mathematical inevitability. In routine used of this method, however, measurement of either of these variables would complicate the procedure to the point of practical uselessness. No large-scale studies are available for more accurate prediction of dead space based upon some such factor as body surface area (which would be essential in children). The possible effects of poliomyelitis upon the dead space have not been quantitatively studied. The effect of tracheotomy upon the dead space has not been assessed. In addition, the problem of a suitable substitute for a mouthpiece in a tracheotomized patient would have to be solved.

The approach suggested by Behrmann[10] is applicable to this problem and requires, besides a Beckman analyzer and end-tidal sampling device, only a soda lime chamber and an apparatus such as that shown in Fig. 1 for collecting a sufficiently large pooled sample of end-tidal air. The P_{CO_2} of a mixture containing CO_2 and O_2 can be calculated if one observes the rise in P_{O_2} which follows removal of CO_2 with soda lime. According to Behrmann, the accuracy of the method lies within 1 mm. Hg P_{CO_2}. It must be emphasized that moment-to-moment variation in end-tidal gas tensions which occurs even in respirator patients is sufficient to invalidate the method unless P_{O_2} is measured before and after CO_2 absorption on the same pooled sample of end-tidal air. Collection of 50 or 100 ml. in a syringe, while attractively simple, is not adequate for the purpose. An apparatus for collecting a larger sample, such as that depicted in Fig. 1, *B*, must be used.

Any clinical reliance placed upon alveolar carbon dioxide measurements presupposes that the arterial to alveolar gradient for the gas is near zero. This is so in the normal lung, but may not be so in the case of pneumonia, atelectasis, or congestion.

SUMMARY

The practical value of an easy, indirect method for measuring end-tidal carbon dioxide tension in respirator patients is noted.

Two indirect approaches, based, respectively, on measurement of end-tidal oxygen tension and of ventilation and oxygen consumption, have been examined in normal subjects and one respirator patient. The first approach is sufficiently accurate to merit clinical trial in the hope of recommending it for small institutions without elaborate facilities. The second approach, while requiring simpler apparatus, is of doubtful value.

A third indirect approach of greater accuracy, described by Behrmann, is suggested as being applicable to the problem, and an apparatus described for collecting sufficient end-tidal air to permit its use.

Dr. Herman Rahn contributed the original suggestion for this work, and was kind enough to furnish much valuable advice. Mrs. Julia N. Gooding prepared the manuscript.

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